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Detection of Anti-Biofilm Activity of *Peppermint* Essential Oil in *S. aureus*, *E. coli* and *P. aeruginosa*

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Abstract Microbial biofilms pose a public health problem and are difficult to eliminate with conventional antimicrobial treatments. This study aimed to detect the anti-biofilm activity of natural *peppermint* essential oil against biofilm-forming bacterial strains. Sixty (60) strains, including *P. aeruginosa* (20), *S. aureus* (20), and *E. coli* (20), from clinical and food sources, were included in this study. The antibacterial activity of *peppermint* essential oil was determined by the Muller Hinton agar diffusion method. Phenotypic biofilm detection and the anti-biofilm activity of *peppermint* essential oil were performed using the tube method. Molecular detection of biofilm-forming genes (*PelA, PslA, and ppyR*) was carried out using PCR. *Peppermint* essential oil exhibited bactericidal activity against foodborne and clinical strains of *E. coli* and *S. aureus*. The median number of biofilms formed ranged from 0.8 ± 0.4 to 1.4 ± 0.4 (clinical strains) and from 0.7 ± 0.4 to 1.1 ± 0.2 (foodborne strains). The biofilm-forming genes PelA, PslA, and ppyR were detected in *P. aeruginosa* with prevalences ranging from 40% to 80% (clinical strains) and from 40% to 60% (foodborne strains). The prevalence of the PelA and PslA genes in *S. aureus* and *E. coli* ranged from 20% to 40% (clinical strains) and from 10% to 40% (foodborne strains). A significant reduction of between 101.7% and 274.8% ($P<0.01$) was observed in clinical and food strains at Raw *peppermint* (RP) doses ranging from 0.2% to 2%. At 0.1% of Raw *peppermint* (RP), a negligible increase of between 21.2% and 23.3% was observed in clinical strains of *P. aeruginosa* and *S. aureus*. Controlling and eliminating bacterial biofilms using local natural essential oil represents a significant therapeutic advance.

Keywords: *Biofilm, anti-biofilm activity, Peppermint, P. aeruginosa, S. aureus and E. coli*

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1. Introduction

Bacterial biofilms pose a significant challenge in many sectors, particularly in the food industry and hospitals [1,2]. They are defined as organized microbial communities adhering to surfaces and enveloped in a protective extracellular matrix [3,4]. This lifestyle of microorganisms makes them particularly resistant to conventional antimicrobial agents and disinfection processes [5,6]. This increased resistance of bacteria in biofilms contributes to the persistence of contamination in food production chains, thus compromising the sanitary quality of products [5,7].

Several studies indicate that the lifestyle, structure, and composition of biofilms lead to increased resistance to antimicrobial agents [5,8]. Among the bacterial species involved in biofilm formation, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli* are increasingly cited [9]. These opportunistic pathogens play a role in various aspects of public health insecurity, from biofilm formation and food poisoning to nosocomial and community-acquired infections [10].

In the food industry, biofilms are the source of numerous problems, both in terms of hygiene and the alteration of the organoleptic qualities of food products [11].

In medicine, biofilms are of particular importance because they are implicated in a wide range of human infections [11]. Approximately 65% of infections in developed countries are due to biofilms. Furthermore, nearly 80% of chronic bacterial infections are associated with the presence of biofilms [11,12]. Infections resulting from biofilms pose significant public health problems [13].

In Côte d’Ivoire, studies have been conducted on the virulence, resistance, and biofilm formation potential of multidrug-resistant strains of animal origin [14,15]. Other studies in Côte d’Ivoire have highlighted the biofilm formation potential of multidrug-resistant clinical strains producing metallo-β-lactamase [16]. Despite these numerous studies, antibiotic therapy remains ineffective for many diseases. Consequently, interest in natural and sustainable alternatives should increase [17,18].

Among anti-infective agents, certain natural essential oils such as those of *peppermint* and propolis exhibit promising therapeutic properties for combating the emergence of resistance and the formation of bacterial biofilms [17,19,20]. *Peppermint* (*Mentha* × *piperita L.*), in particular, is used in various forms, including essential oils, leaves, and extracts, and is incorporated into food, cosmetics, and pharmaceuticals [17].

Indeed, *peppermint* (*Mentha* × *piperita L.*) possesses purifying, antibacterial, anti-inflammatory, soothing, and healing properties. These various anti-infective potentials of this natural substance are linked to its composition, structure, and intrinsic properties [14,15].

In Côte d’Ivoire, the effect of effective natural plant products on the simultaneous formation of bacterial biofilms from food and hospital sources remains less well documented. In this context, the present study aims to evaluate the anti-biofilm activity of *peppermint* oil.

2. Materials and Methods

2.1. Biological Materials

Figure 1. Peppermint ; A: Peppermint oil; B: Peppermint leaf

The strains used in this study consisted of clinically and foodborne strains of *P. aeruginosa* (20), *S. aureus* (20), and *E. coli* (20) isolated from previous studies and stored at -20°C. The reference strains of *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 25923, and *E. coli* ATCC 25922 from the collection of the Pasteur Institute, Abidjan, Côte d'Ivoire, were used as controls for antibiotic resistance testing. The plant material used was *peppermint* essential oil (Figure 1).

2.2. Methods

2.2.1. Bacterial Isolates

A total of sixty (60) bacterial strains, composed of *P. aeruginosa* (20), *S. aureus* (20), and *E. coli* (20), were studied (Table 1). The strains were obtained from clinical samples (blood, stool, pus, and urine) isolated at the Pasteur Institute of Côte d'Ivoire, as well as from food samples (smoked fish, fresh fish, and beef). These *P. aeruginosa*, *S. aureus*, and *E. coli* strains were isolated on Cetrimide, *Staphylococcus* agar, and TBX agar, respectively. These bacterial strains isolated on selective media underwent biochemical and molecular identification by PCR.

Table 1. Diversity of bacterial strains used

Origin of the strains	Type of strains	Number (n)
Clinics	*P. aeruginosa*	10
	S. aureus	10
	E. coli	10
Food	*P. aeruginosa*	10
	S. aureus	10
	E. coli	10
Total	Strains	60

2.2.2. Molecular Characterization of Bacterial Isolates

Molecular identification by polymerase chain reaction (PCR) was performed on different strains of *P. aeruginosa*, *E. coli*, and *S. aureus*. The operation was carried out in several steps, including the extraction of genomic DNA, the amplification of the 16S gene characteristic of the bacterial species (*P. aeruginosa*, *E. coli*, and *S. aureus*), and the visualization of the amplification products.

2.2.2.1. Extraction and Purification of Genomic DNA

All isolated strains of *P. aeruginosa*, *S. aureus* and *E. coli* were revived in brain-heart broth (BHC) for 24 h. The genomic DNA of these strains was then extracted by the thermal lysis method and purified according to the technique described by Al-Kilabi et al. [21]. After extraction, the DNA was diluted and stored at -20°C to serve as a template for polymerase chain reaction (PCR).

2.2.2.2. Preparation of the Reaction Mixture (Mix)

The reaction mixture was prepared according to the technique described by Al-Kilabi et al. [21]. This reaction mixture with a total volume of 25 µL consisted of 15 µL of sterile Milli-Q water (milli-Q™, Millipore Corporation, USA), 5 µL of 5X concentration loading buffer, 1.5 µL of MgCl2, 2 mM (Promega Corporation, Madison, WI 53711-5399, USA), 0.2 µL of 10 mM dNTPs, 0.1 µL of each primer, 10 mM (Integral DNA Technology, California, USA), 0.1 µL of Go Taq® G2 Flexi DNA polymerase with a final concentration of 1.5U (Promega

Corporation, Madison, WI 53711-5399, USA) and 3 µL of the DNA template.

Reference strains and sterile Milli-Q water were used respectively as positive control and negative control for each step of the PCR reaction.

2.2.2.3. 16S rDNA Amplification

Amplification of the 16S rDNA gene was performed according to the technique described by Yehia et al. [22] using the different primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (3'TACGGYTACCTTGTTACGACTT-5') (Table 2). The amplification program consisted of an initial 5-min denaturation step at 94°C followed by 35 cycles comprising denaturation (94°C for 30 s), hybridization (55°C for 40 s), and extension (72°C for 30 s), with a single final 10-min extension at 72°C. All samples were stored at +4°C until the thermocycler was shut down.

2.2.2.4. Amplification of Biofilm Formation Genes

Amplification of the genes responsible for biofilm formation (*pslA*, *pelA*, *ppyR*) was performed according to the method described by Yehia et al. [22]. The amplification program consisted of an initial 5-min denaturation at 95°C followed by a repeated cyclic phase. The cyclic phase, repeated 33 times with each amplification cycle, comprised a 30-second denaturation step at 95°C, a 60-second primer binding (hybridization) step at 65°C, and a 90-second elongation step at 72°C. The amplification reaction concluded with a final 5-min elongation at 72°C. The amplification programs and the nucleotide sequence of the primers used for PCR are described in the Table 2.

2.2.2.5. Electrophoresis of Amplification Products

Gene amplification products were visualized on a 1.5% agarose gel after 120 V incubation for 30 min and visualized by illumination on a UV plate of a light source and photographed (Molecular Imager Gel Doc™ EZ, Bio-Rad, USA) as described previously. A 4 µL volume of a molecular weight marker (Bench Top, 1 kb DNA Ladder, Promega Corporation, USA) introduced into the first well of the solidified gel was used as a reference scale.

Table 2. Primers used for identification and biofilm formation

Genes	Primer sequence (5′→3′)	Amplification program	Size (bp)	References
Identification : 16S	27 F : 5’- AGAGTTTGATCMTGGCTCAG -3’ 1492 R : 5’- TACGGYTACCTTGTTACGACTT-3’	94°C, 5 min 35 x [94°C, 30 s; 55°C, 30s; 72°C,1min] 72°C, 10 min; 4°C.	1500	[23]
Biofilm : *pslA*	F : 5’-TCCCTACCTCAGCAGCAAGC-3’ R : 5’-TGTTGTAGCCGTAGCGTTTCTG-3’	95°C, 5 min 33 x [95°C, 30 s; 65°C, 60s; 72°C, 90s] 72°C, 5 min; 4°C.	656	[24]
PelA	F : 5’-CATACCTTCAGCCATCCGTTCTTC-3’ R : 5’-CGCATTCGCCGCACTCAG-3’		786	
PpyR	F : 5’-CGTGATCGCCGCCTATTTCC-3’ R : 5’-ACAGCAGACCTCCCAACCG-3’		160	

Pel: dandruff (Pel gene encoding dandruff); Psl: polysaccharide synthesis locus (polysaccharide synthesis); ppyR: regulator of the psl and pyoverdine operon, PA2663 (Regulator of the psl and pyoverdine operons)

2.2.3. Evaluation of the Antibacterial Activity of Raw *peppermint* Essential Oil

The antibacterial activity of *peppermint* essential oil is expressed by the appearance of inhibition zones around the discs. It is assessed by measuring the diameter of the inhibition zone produced around the discs using calipers or a ruler.

2.2.3.1. Determination of Minimum Inhibitory Concentrations of Peppermint Essential Oil

By definition, the minimum inhibitory concentration (MIC) is the lowest concentration at which an essential oil is capable of inhibiting the development or growth of a particular microorganism. The MIC of an extract against a given strain will be the lowest concentration showing no visible growth of the microorganism.

The various MICs were determined in liquid medium in sterile hemolysis tubes. A range of concentrations of the essential oil to be tested was prepared in seven hemolysis tubes using the double dilution method in liquid medium. Ten milliliters of essential oil concentrated at 0.2 mL/mL were prepared and sterilized in an autoclave at 121°C for 15 minutes. For each essential oil, a range of sterile concentrations of 0.2, 0.1, 0.05, 0.025, 0.0125, and 0.00625 mL/mL was prepared in hemolysis tubes. Also for each bacterial strain, an inoculum of 108 bacteria/mL was prepared in twice concentrated Mueller-Hinton broth.

Next, using the double dilution method, 1 mL of bacterial inoculum was added to each tube of the essential oil range. The concentration range of each essential oil was then diluted by half, resulting in the following concentrations: 0.1, 0.05, 0.025, 0.0125, 0.00625, and 0.003125 mL/mL. A positive control tube containing 1 mL of sterile distilled water and 1 mL of inoculum, and a negative control tube containing 1 mL of sterile distilled water and 1 mL of sterile Mueller-Hinton broth, were also prepared. The prepared tubes were incubated at 37°C for 24 hours. After incubation, bacterial growth in each tube, indicated by turbidity of the medium, was observed.

2.2.3.2. Determination of the Minimum Bactericidal Concentration of *peppermint* Essential Oil

The minimum bactericidal concentration (MBC) is defined as the smallest dose capable of killing the microorganism. After inoculating the tubes during the MIC reading, a bactericidal control was prepared from the inoculum. Using a calibrated 10 µL platinum loop, dilutions of 10^{-1}, 10^{1}, 10^{-2}, 10^{-3}, and 10^{-4} of the inoculum, corresponding to 100%, 10%, 1%, 0.1%, and 0.01% of bacteria in suspension, respectively, were inoculated in strips onto Mueller-Hinton agar in Petri dishes. The Petri dishes were incubated at 37°C for 24 hours.

After reading the MIC, subculturing in strips from tubes without visible growth was performed onto Petri dishes containing Mueller-Hinton agar. These plates were then incubated at 37°C for 24 h. After incubation, the strips were compared to the bactericidal control. In practice, the MBC corresponds to the lowest concentration at which subculturing shows bacterial growth less than or equal to the number of colonies observed at the 10^{-4} dilution (0.01% survivors).

2.2.3.3. Evaluation of the Bactericidal or Bacteriostatic Effect of *peppermint* Essential Oil

The CMB/MIC ratio helps to define the mode of action of an essential oil. When the CMB/MIC ratio is less than or equal to 2, the essential oil is considered bactericidal. Conversely, if this ratio is greater than 2, the essential oil is considered bacteriostatic.

2.2.4. Anti-biofilm Activity of *peppermint* Essential Oils

A range of graduated concentrations was used to study the effect of crude *peppermint* essential oils (CPOs) on biofilm formation in *P. aeruginosa*, *E. coli*, and *S. aureus*. *Peppermint* essential oil was aseptically added to Luria Bertani broth (LB) at the following concentrations: 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 1%, 1.5%, and 2% (v/v) in the presence of three (3) drops of Twens Twens 80.

2.2.4.1. Preparation of Initial Bacterial Suspensions

To perform this test, each bacterial strain was first inoculated onto Luria-Bertani (LB) agar. The plates were then incubated at 37°C for 24 hours. After 24 hours, a few colonies were suspended in LB broth free of *peppermint* oil (LB Control). Next, a few colonies were also suspended in LB broth with varying concentrations of *peppermint* oil added (LB Test). Finally, all optical densities at 600 nm (OD) of the resulting suspensions were adjusted to 0.20 using a spectrophotometer.

2.2.4.2. Biofilm Formation Tests in Tubes

The bacterial suspensions thus prepared were dispensed into 5 mL polystyrene tubes, with 2 mL of suspension per tube. The tubes were then incubated at 37°C for 48 hours under static conditions.

2.2.4.3. Quantification of Biofilms Formed

After 48 hours of incubation, the tubes were removed, and the absorbance of the resulting bacterial culture was measured at 600 nm for each tube. The bacterial culture was then removed by successive rinsings with distilled water (DW). Biomass attached to the tube walls (biofilm formation) was revealed after staining with a 1% aqueous solution of crystal violet (CV). Two mL volumes of 1% (v/v) crystal violet were dispensed into each tube. The tubes were incubated for 30 minutes at room temperature. After a contact time of 1 hour, excess stain was removed by 3 to 10 successive, thorough manual washes of the tube walls with sterile distilled water (DW) (until clear droplets were obtained). The CV (color transferase) stain, which had adhered to the tube walls, was solubilized using a 75:25 ethanol-acetone solution for 1 hour.

The stain, having penetrated the biofilm bacteria and adhered to the tube walls, was then solubilized for 1 hour by adding 2 mL of 95% absolute ethanol to each tube. The absorbance of the resulting solution was then measured at 570 nm using a spectrophotometer.

Finally, the strains were grouped into: OD 570 < 0.1, non-biofilm producers (NP); OD 570 = 0.1–1.0, weak biofilm producers (WP); OD 570 = 1.1–3.0, moderate biofilm producers (MP); and OD 570 > 3.0, strong biofilm producers (SP).

2.2.4.4. Determining the Percentage Increases or Reductions in Biofilms

In the case of a possible increase in bacterial adhesion or planktonic growth, the results are expressed as a percentage increase in biofilms. It is calculated as follows:

$$\textbf{Percentage increase}\left(\%\right)=\frac{\text{DO Test}-\text{DO Control}}{\text{DO Control}}\text{X100}$$

Dans le cas d'une éventuelle inhibition de la formation de biofilms ou de la croissance des cellules non fixées, les résultats sont exprimés en pourcentage de réduction de biofilms. Il est calculé comme suit:

$$\textbf{Percentage reduction}\left(\%\right)=\frac{\text{DO Control}-\text{DO Test}}{\text{DO Control}}\text{X100}$$

2.2.5. Statistical Analyses

The data underwent both manual and computer processing. SPSS 20.0 software was used to analyze the data. Excel was used to determine frequencies, means, and standard deviations.

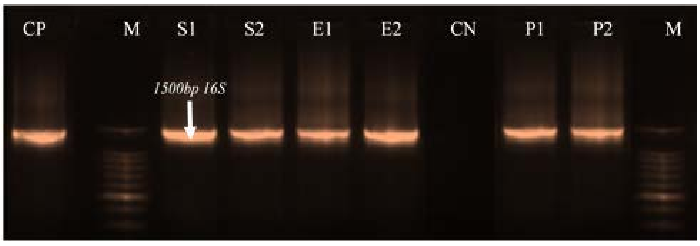
3. Results

3.1. Identified Bacterial Strains

Phenotypic, biochemical, and molecular identification using the 16S marker (Table 3) allowed the selection of sixty (60) strains, including *P. aeruginosa* (20), *E. coli* (20), and *S. aureus* (20), from a set of ninety (90) isolates from clinical and food samples. Electrophoretic profiling detected and confirmed the presence of *S. aureus, E. coli*, and *P. aeruginosa* strains (Figure 2).

Table 3. Confirmed bacterial strains

Identification	Foodborne and hospital germs			Number (n)	Percentage (%)
	P. aeruginosa (n = 30)	*E. coli* (n = 30)	*S. aureus* (n = 30)	90	100 %
Phenotypic	24	26	26	76	84.4%
Biochemical	21	23	24	68	75.5%
Molecular	20	20	20	60	66.6%



*CP: Positive Control; CN: Negative Control; M: Molecular weight marker, S1-S*2 presence of S. aureus; E1-E2 presence of E. coli; P1-P2 presence of P. aeruginosa. (Bench Top, 1500 bp DNA Ladder, Promega Corporation, USA).

Figure 2. Electrophoretic profile of the 16S rRNA gene of the studied organisms

3.2. Biofilm Formed and Categories of Producers

The study demonstrated the phenotypic potential of clinical and foodborne strains of *P. aeruginosa*, *E. coli*, and *S. aureus* to form biofilms. The median number of biofilms formed ranged from 0.8 ± 0.4 to 1.4 ± 0.4 (clinical strains) and from 0.7 ± 0.4 to 1.1 ± 0.2 (foodborne strains) (Table 4). The biofilm-producing categories showed that some strains were non-producers (NP). Other strains produced biofilms, classified as high producers (FP), moderate producers (PM), and low producers (fP).

The high-producer (22% to 32%) and moderate-producer (40% to 58%) categories were significantly higher in clinical strains (Table 4). The results indicated that clinical and food strains of *E. coli* have the same level of biofilm formation with a median of 0.8 ± 0.4 (Table 4).

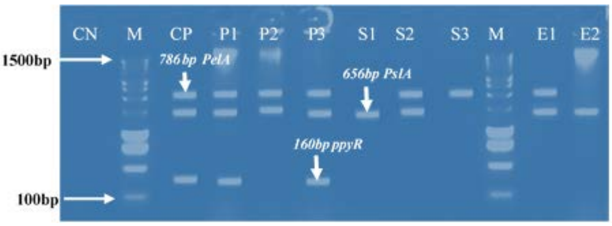
Table 4. Median biofilm and producer categories

Type of Strains		Biofilm formed after 48 hours			Category of producers of biofilms after 48 hours			
		Minimum OD	Median OD	Maximum OD	NP	f P	PM	FP
Clinics	*P. aeruginosa*	0.5	1.4 ± 0.4	3.5	2%	8%	58 %	32 %
	S. aureus	0.3	1.1 ± 0.3	2.1	8%	25 %	38 %	29 %
	E. coli	0.2	0.8 ± 0.4	1.5	10%	28 %	40 %	22 %
Food	*P. aeruginosa*	0.3	1.1 ± 0.2	1.7	13%	18 %	37 %	32 %
	S. aureus	0.2	0.7 ± 0.4	1.1	15%	35 %	27 %	23 %
	E. coli	0.2	0.8 ± 0.4	1.4	20%	40 %	20 %	20 %

NP: Non-Producer; lP: Low Producer; MP: Moderate Producer; HP: High Producer

3.3. Biofilm Formation Genes in *P. aeruginosa*, *S. aureus* and *E. coli*

Figure 3 present the electrophoretic profiles of genes involved in biofilm formation in *P. aeruginosa*, *S. aureus*, and *E. coli*. Some *P. aeruginosa* strains expressed three (3) biofilm-forming genes (*PelA*, *PslA*, and *ppyR*), while others had only one or two (Figure 3). The biofilm-forming genes *PelA*, *PslA*, and *ppyR* were detected in *P. aeruginosa* with prevalences ranging from 40% to 80% and 40% to 60% in clinical and foodborne strains, respectively (Table 5). *S. aureus* and *E. coli* strains harbored two (2) or one (1) biofilm-forming genes (*PelA* and/or *PslA*) (Figure 3). The prevalence of the *PelA* and *PslA* genes in *S. aureus* and *E. coli* ranged from 20% to 40% (clinical strains) and from 10% to 40% (foodborne strains). No gene encoding pyoverdine production (*ppyR*) was detected in *S. aureus* or *E. coli* (Table 5).



In P. aeruginosa : *Wells P1 and P3: presence of all three genes (PelA, PslA, and ppyR); well P2: presence of two genes (PslA and ppyR).* ***In S. aureus :*** *Wells S1: presence of one gene (PslA) ; Wells S2: presence of two genes (PelA, PslA) ; Wells S3 : presence of one gene (PelA).* ***In E. coli :*** *Wells E1: presence of two genes (PelA, PslA) ; Wells E2 : presence of one gene (PslA). CP: Positive Control, CN: Negative Control. M: Molecular weight marker*

Figure 3. Electrophoretic profile of genes involved in biofilm formation

Table 5. Prevalence of Biofilm Genes

Type of Strains		Prevalence of Biofilm Genes		
		PslA	*PelA*	*PpyR*
Clinical strains	*P. aeruginosa* (n=10)	70,0 % (7)	40,0 % (4)	80,0 % (8)
	S. aureus (n=10)	40,0 % (4)	20,0 % (2)	0,0 % (0)
	E. coli (n=10)	40,0 % (4)	30,0 % (3)	0,0 % (0)
Food strains	*P. aeruginosa* (n=10)	60,0 % (6)	40,0 % (4)	60,0 % (6)
	S. aureus (n=10)	30,0 % (3)	10,0 % (1)	0,0 % (0)
	E. coli (n=10)	30,0 % (3)	30,0 % (3)	0,0 % (0)

Pel: dandruff (Pel gene encoding dandruff); Psl: polysaccharide synthesis locus (polysaccharide synthesis); ppyR: regulator of the psl and pyoverdine operon, PA2663 (Regulator of the psl and pyoverdine operons)

3.4. Antibacterial Activity of *Peppermint* Essential Oil

Analysis of the MBC/MIC ratio results shows that *peppermint* essential oil is bactericidal against foodborne and clinically acquired *E. coli* and *S. aureus* strains with MBC/MIC ratios less than or equal to two (2). As for *P. aeruginosa* strains, they exhibit bactericidal and bacteriostatic activity against foodborne and clinically acquired strains, respectively (Table 6).

Table 6. Prevalence of Biofilm Genes

Bacterial strains	*Peppermint essential oil*					
	Food Strains			Clinical Strains		
	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC
P. aeruginosa	0.05	0.1	2	0.025	0.1	4
S. aureus	0.0125	0.025	2	0.025	0.05	2
E. coli	0.0125	0.025	2	0.05	0.05	1

MIC: Minimum Inhibitory Concentration; MBC: Minimum Bactericidal Concentration

3.5. Anti-biofilm Activity of Raw *peppermint* (RP)

With Raw *peppermint* (RP) concentrations between 0.2% and 2%, the results showed no increase in the adhesion capacity of clinically and foodborne strains (Table 7). At a dose of 0.1% Raw *peppermint* (RP), a statistically insignificant increase of between 21.2% and 23.3% was observed in clinical strains of *P. aeruginosa* and *S. aureus* (Table 7). The effect of raw *peppermint* (RP) shows that the median biofilms reflecting these different increases range from 1.5 to 1.7 (Figure 4).

Furthermore, the results show, for both clinical and food strains, a reduction exceeding 100% ranging from 101.7% to 274.8% which is statistically very significant (P<0.01) for Raw *peppermint* (RP) doses ranging from 0.2% to 2% (Table 7).

Table 7. Biofilm evolution in the presence of raw *Peppermint* (RP)

Origin of Strains		Concentrations in Raw *peppermint* (RP) (%)							
		0.1	0.2	0.3	0.4	0.5	1	1.5	2
Percentage increase in biofilm (%)									
Clinical strains	*P. aeruginosa*	21.2	ϕϕ	ϕϕ	ϕϕ	ϕϕ	ϕϕ	Φϕ	Φϕ
	S. aureus	23.3	ϕϕ	ϕϕ	ϕϕ	ϕϕ	ϕϕ	Φϕ	Φϕ
	E. coli	ϕϕ	ϕϕ	ϕϕ	ϕϕ	ϕϕ	ϕϕ	Φϕ	Φϕ
Food strains	*P. aeruginosa*	ϕϕ	ϕϕ	ϕϕ	ϕϕ	ϕϕ	ϕϕ	Φϕ	Φϕ
	S. aureus	ϕϕ	ϕϕ	ϕϕ	ϕϕ	ϕϕ	ϕϕ	Φϕ	Φϕ
	E. coli	ϕϕ	ϕϕ	ϕϕ	ϕϕ	ϕϕ	ϕϕ	Φϕ	Φϕ
Percentage of biofilm reduction (%)									
Clinical strains	*P. aeruginosa*	ϕ	101.7	118.1	150.2	178.5	192.3	2057	235.4
	S. aureus	ϕ	120.4	128.8	154.2	165.5	171.1	207.8	254.2
	E. coli	32.6	118.6	1203	165.6	195.4	242.1	258.5	262.1
Food strains	*P. aeruginosa*	23.8	104.2	108.1	157.7	185.2	207.3	224.1	257.5
	S. aureus	32.2	102.1	108.2	162.1	173.5	186.4	232.3	274.8
	E. coli	52.4	108.2	137.7	198.1	210.4	258.6	261.7	274.1

(ϕϕ : No increase, ϕ: No reduction)

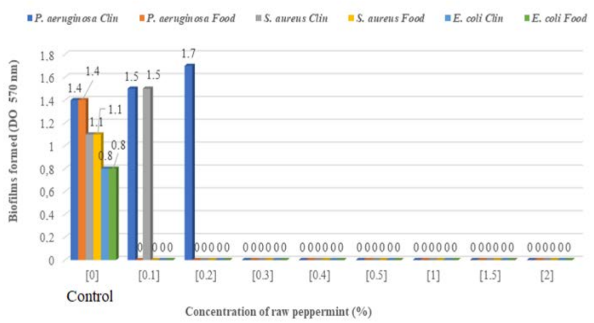


Figure 4. Raw Peppermint concentration (%)

4. Discussion

This work enabled the phenotypic and molecular detection of genes responsible for bacterial biofilm formation in *P. aeruginosa*, *S. aureus*, and *E. coli*, leading to improved diagnosis and management using natural substances. Bacterial biofilms represent a significant public health problem affecting numerous sectors, including the agri-food industry and hospitals [2,5]. Biological diagnosis of these biofilm-forming bacterial infections, based on phenotypic methods, is limited in favor of molecular methods [25]. In this work, the 16S marker enabled molecular identification of *S. aureus*, *E. coli*, and *P. aeruginosa* involved in biofilm formation using primers 27-F and 1492-R [16,25]. Several similar studies have indicated that the 16S gene provides high resolution in the molecular identification of bacteria [25].

Furthermore, the phenotypic potential of clinical and foodborne strains of *P. aeruginosa*, *E. coli*, and *S. aureus* to form biofilms showed a median number of biofilms formed ranging from 0.8 ± 0.4 to 1.4 ± 0.4 (clinical strains) and from 0.7 ± 0.4 to 1.1 ± 0.2 (foodborne strains). In addition to phenotypic detection, these strains harbored a diversity of genes (*PelA*, *PslA*, and *ppyR*) involved in biofilm formation, conferring antimicrobial resistance [1,4,6]. These molecular supports for biofilm formation could justify multi-resistance at the level of different clinical and food strains [2,7,26].

Indeed, numerous studies have shown that biofilm-forming bacteria can become 10 to 1000 times more resistant to antimicrobial agents than their planktonic counterparts of the same strain [27]. Consequently, combating these biofilms responsible for multidrug resistance represents a significant microbiological and medical challenge. Thus, the development of new strategies and the discovery of new targets are clearly essential to combat bacterial biofilms [26,28]. Anti-infective agents that selectively disrupt virulence pathways to prevent or cure infection are less likely to promote the emergence of resistance [1,3,28]. Among these anti-infective agents, some natural essential oils have promising therapeutic properties to combat bacterial biofilms [17,18].

This study investigated the potential effects of raw *peppermint* (RP) essential oil on the adhesion of *P.*

aeruginosa, *S. aureus*, and *E. coli* [17]. This natural plant product was tested in its raw form at various concentrations on clinical and foodborne strains. At a dose of 0.1% raw *peppermint* (RP), a statistically insignificant increase of between 21.2% and 23.3% was observed in clinical strains of *P. aeruginosa* and *S. aureus*.

Furthermore, the results showed a statistically significant reduction ($P<0.01$) of between 101.7% and 274.8% for both clinical and foodborne strains, with Raw *peppermint* (RP) concentrations ranging from 0.2% to 2%.

Therefore, *peppermint* oil, with its known characteristics, exhibited a bactericidal and anti-biofilm effect, capable of modifying biofilm formation kinetics and significantly reducing the number of adherent cells [17,29]. These results are similar to those of Benie et al. (2021), who indicated in their study that, with characteristics similar to furanone, *peppermint* oil exhibited an inhibitory effect on biofilm formation and quorum sensing (QS) activity.

The bactericidal and anti-biofilm efficacy of this oil in eliminating and dispersing bacterial biofilms has shown that this molecule may act on the various communication systems involved in quorum sensing in bacteria [29,31] (Rosignoli et al., 2026; Wang et al., 2025; Maisuria et al., 2016). A toxicity study could define the potential benefit of *peppermint* oil in the preventive or curative treatment of bacterial biofilm infections.

5. Conclusion

Strains of *P. aeruginosa*, *E. coli*, and *S. aureus* exhibited phenotypic and molecular potential to form biofilms. The biofilm-forming genes *PelA*, *PslA*, and *ppyR* were predominantly detected in *P. aeruginosa*. This study demonstrated that *peppermint* essential oil is effective in eradicating biofilms of *P. aeruginosa*, *E. coli*, and *S. aureus*. This natural essence may be a promising candidate for treating bacterial biofilms in the food industry and in hospital settings.

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