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Prevalence of Biofilm Formation and *Quorum Sensing* Genes in *Pseudomonas aeruginosa* and *Escherichia coli* Isolated from Smoked Fish

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This investigation sought to ascertain the ability of Pseudomonas aeruginosa and Escherichia coli isolated from contaminated smoked fish to form biofilms and engage in quorum sensing. Sixty strains of Pseudomonas aeruginosa and forty strains of Escherichia coli isolated from smoked fish were used to conducted this study. The strains were first revived and then inoculated onto Cetrimide and Tryptone bile X glucuronide media. Quorum sensing (LasI and LasR) and biofilm formation gene such as ppyR, PelA and PslA were detected by PCR methods. In P. aeruginosa strains, genes Las were detected with a prevalence of 75% for LasI and LasR. The prevalence of Rhl genes was 58.33% for RhlI and 50% for RhlR, respectively. In E. coli strains, the prevalence was 71.42% for the genes (RhlI/R) and 42.85% for the genes (LasI/R). The biofilm-forming genes PslA, ppyR and PelA were detected in Pseudomonas aeruginosa strains with respective percentages of 50%, 41.66%, and 58.33%. Only PelA (14.28%) and PslA (28.57%) were detected in Escherichia coli strains. Pseudomonas aeruginosa and Escherichia coli are involved in biofilm formation and quorum sensing. Smoking techniques and smoked fish preservation methods need to be improved.

Keywords: Biofilm; Escherichia coli; Pseudomonas aeruginosa; Quorum sensing; Smoked fish.

Biofilms are complex and interconnected bacterial communities. They can adhere to various surfaces in the food industry, kitchens (cutting boards, plumbing fixtures), and even on equipment used in hospitals.[1] Extracellular polymeric substances (EPS), proteins, lipids, and extracellular DNA are the most important components in the formation of the biofilm's complex matrix. Biofilm shielded bacteria from antimicrobial agents, host immunological responses, and adverse surroundings.[2-4] Biofilm formation is considered a bacterial survival strategy in hostile environments.[5] A matrix of extracellular polymeric materials envelops and shields the bacterial communities residing in biofilms. The bacterial species and ambient conditions determine the physicochemical

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characteristics and makeup of these extracellular polymeric materials.[6]

Food contamination and spoiling can result from pathogenic bacteria like *Escherichia coli* and *Pseudomonas aeruginosa* growing and sticking to the surfaces of tools and utensils. These bacteria are commonly found in fish. Their participation in adhesion processes is well-known, biofilm formation, and quorum sensing in humans and animals.[3,7] Furthermore, they are implicated in nosocomial infections and food poisoning through a diversity of virulence factors mediated by quorum sensing (QS).[8,9]

Biofilms have a major impact on the pathogenicity and antibiotic resistance of *Pseudomonas aeruginosa*. *Pseudomonas aeruginosa* needs type IV pili and a single polar flagellum in order to attach, colonize surfaces, and form biofilms. He can survive on biotic and abiotic surfaces while eluding human immune responses thanks to biofilms.[10] *Pseudomonas aeruginosa* associated with biofilms exhibits increased antibiotic resistance.[11-13]

Escherichia coli is not naturally present in the fish microbiota but can be introduced from contaminated aquatic environments or during fish handling.[14] The presence of *Escherichia coli* in food, including fish, is considered an indicator of faecal contamination.[15] Although most *Escherichia coli* strains are non-pathogenic, various intestinal serotypes are opportunistic pathogens and can cause gastrointestinal, urinary, or central nervous system diseases.[16] *Escherichia coli* possesses both the ability to form biofilms and antibiotic resistance.[17,18] Consequently, they can survive for many years in food-processing environments.[19]

The increase in antimicrobial resistance among food-producing animals, including seafood, may aid in the food chain's spread to humans.[20,21] Certain pathogens' capacity to build biofilms makes treatment management even more difficult. Biofilms act as physical barriers, significantly reducing the effectiveness of antibiotics and immune recognition.[22] The presence of pathogenic bacteria in biofilms that form on the contact surfaces of fumigation ovens and display counters poses a potential risk of disease transmission to consumers.[23]

Acting as physical barriers, biofilms markedly diminish the efficacy of antibiotics and impair immune recognition.[22] When pathogenic bacteria are embedded within biofilms on food-contact surfaces (such as smoking ovens or fish display counters) they can serve as persistent reservoirs of contamination and contribute to human infections.[23]

Biofilms formed from hospital and animal strains have been studied in Côte d'Ivoire.[24] However, Research on quorum sensing and biofilm formation in smoked fish is virtually non-existent. The overarching objective of this study was to determine the genetic potential for *quorum sensing* and biofilm formation in *Escherichia coli* and *Pseudomonas aeruginosa* isolated from smoked fish sold in markets across Abidjan.

MATERIALS AND METHODS

Materials

Biological Material

The biological material consisted of multidrug-resistant strains of *Pseudomonas aeruginosa* and *Escherichia coli*, isolated from smoked fish. These strains originated from a previous study.[25]

Methods

Confirmation of strains

Colonies characteristic of *Pseudomonas aeruginosa* and *Escherichia coli* were streaked onto selective and non-selective agar plates. Gram staining, the fresh-state test, and the classic LEMINOR miniature gallery test were performed on presumptive *Escherichia coli* colonies after 18 to 24 hours of incubation at 37°C.

After 24 hours of growth on Luria-Bertani Agar (LB), the oxidase-positive strains were cultured on King A and King B media. Those producing both types of pigment: pyoverdin (yellow-green, fluorescent pigment) on King B and pyocyanin (blue, non-fluorescent pigment) on King A, or pyocyanin alone on King A, and capable of growing at 42°C were identified as *Pseudomonas aeruginosa*. Oxidase-positive strains that did not produce pyocyanin and could not grow at 42°C were considered *Pseudomonas* sp.

Preservation of isolates

The identified bacterial strains of *Pseudomonas aeruginosa* and *Escherichia coli* was sub-cultured in duplicate into Tryptic Soy Broth (TSB) (Sigma-Aldrich)-filled hemolysis tubes, followed add 500 µL of glycerol to reach a final concentration of 15%. After 3 hours of growth at 28/ °C, the cultures were preserved transiently for 1 hour at –20/ °C and subsequently stored at –80/ °C.

Identification of genes implicated in the development of biofilms

Amplification of genes: *ppyR*, *pslA*, *pelA*

The technique outlined by Pournajaf et al.[26] was used to amplify the biofilm formation genes (*pslA*, *pelA*, and *ppyR*).[26] The amplification program comprised a repeated cyclic phase after an initial denaturation for five minutes at 95 °C. A denaturation step of 30 seconds at 95°C, a primer annealing step of 60 seconds at 65°C, and an elongation step of 90 seconds at 72°C made up this cyclic phase, which was repeated 33 times. A final elongation of five minutes at 72°C marked the end of the amplification reaction. On a T9700 Thermocycler (Applied Biosystems, China), samples were kept at +4°C until the run was finished. The nucleotide sequences of the primers used are presented in Table 1.

Amplification of *quorum sensing* genes (*LasI*, *LasR*, *rhlI*, *rhlR*)

Quorum sensing genes (*LasI*, *LasR*, *rhlI*, and *rhlR*) were amplified using the procedure outlined by Lima et al.[27] The amplification program comprised a cyclic phase that was repeated 33 times after an initial denaturation for five minutes at 95 °C. Each cycle included a 30-second denaturation step at 95°C, a 60-second primer annealing stage at 65°C, and a 90-second elongation step at 72°C. A final elongation of five minutes at 72°C marked the end of the amplification reaction. Samples were kept on a Thermocycler (Applied Biosystems) at +4°C until the run was finished. The nucleotide sequences of the primers used are presented in Table 1.

Electrophoresis of amplification products

A transilluminator was used to view the bands under UV light after they had migrated at 120 V for 30 minutes on an 8% agarose gel. A Molecular Imager Gel DocTM EZ system (Bio-Rad, USA) was then used to take pictures of them.

Calculation of the prevalence of isolated genes.

To determine the prevalence of individual genes, the following formula was used.

$$P\ (\%) = \frac{number\ of\ gene\ (n)}{number\ of\ each\ strains}\ x\ 100$$

Data analysis

To investigate the possible relationship between the genes involved in biofilm formation and quorum sensing and the isolated bacterial strains, a principal component analysis (PCA) was performed.

Table 1. Primers used for the amplification of *ppyR, pslA*, *pelA* genes and *LasI*, *LasR*, *rhlI*, *rhlR*

	Genes	Primers sequences (5'’!3')	Base pair size
Biofilm	*pslA*	F : 5’-TCCCTACCTCAGCAGCAAGC-3’ R : 5’-TGTTGTAGCCGTAGCGTTTCTG-3’	656
	PelA	F : 5’-CATACCTTCAGCCATCCGTTCTTC-3’ R : 5’-CGCATTCGCCGCACTCAG-3’	786
	PpyR	F : 5’-CGTGATCGCCGCCTATTTCC-3’ R : 5’-ACAGCAGACCTCCCAACCG-3’	160
quorum sensing	*LasI*	F : 5’-CGTGCTCAAGTGTTCAAGG -3’ R : 5’-TACAGTCGGAAAAGCCCAG -3’	295
	LasR	F : 5’-AAGTGGAAAATTGGAGTGGAG-3’ R : 5’-GTAGTTGCCGACGACGATGAAG- 3’	130
	rhlI	F : 5’-TTCATCCTCCTTTAGTCTTCCC-3’ R : 5’-TTCCAGCGATTCAGAGAGC -3’	155
	rhlR	F : 5’-TGCATTTTATCGATCAGGGC-3’ R : 5’-CACTTCCTTTTCCAGGACG-3’	133
	IMP	F : TGAGCAAGTTATCTGTATTC R : TTAGTTGCTTGGTTTTGATG	740

RESULTS

Quorum sensing and biofilm-associated genes detected by PCR

Molecular determinism of *quorum sensing* genes: *LasI, LasR, rhlI, rhlR*

Figure 1 illustrates the electrophoretic profiles of genetic determinants involved in *quorum sensing* in multidrug-resistant *Escherichia coli* and *Pseudomonas aeruginosa*. Certain strains of *Escherichia coli* and *Pseudomonas aeruginosa* harbored the *Las* or *Rhl* genes, which encode elastase and rhamnolipid production, respectively. In *Pseudomonas aeruginosa*, the prevalence of *Las* genes was 77.41% for *Las*I and 77.41% for *Las*R. The prevalence of *Rhl* genes was 58.06% for *Rhl*I and 54.83% for *Rhl*R. In *E. coli*, *Las* and *Rhl* genes were detected with respective prevalences of 38.88% (*Las*I/R) and 72.22% (*Rhl*I/*Rhl*R) (Table 2).

Molecular determinants of biofilm: *ppy*R, *Pel*A and *Psl*A

Figure 2 shows the electrophoretic profiles of biofilm forming genes in multidrug

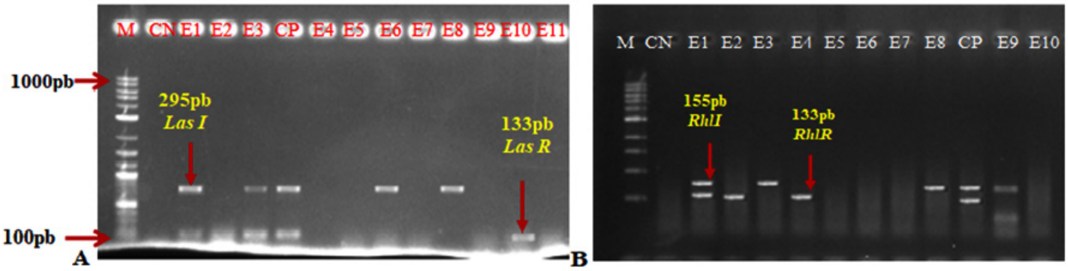


Figure 1A: - *M: Molecular weight marker, - CN: Negative control strain (**E. coli ATCC 25922)***
- E1 through E11: Strains of Pseudomonas aeruginosa and Escherichia coli in which the lastI and lastR genes were screened for.
- E1 and E2: Pseudomonas aeruginosa strains harboring the lastI gene
- E1, E2, and E3: Pseudomonas aeruginosa strains harboring the lastR gene. - E4 and E5: Pseudomonas aeruginosa strains harboring neither the lastI gene nor the lastR gene
- E6 and E8: Escherichia coli strains harboring the lastI gene. - E10: Escherichia coli strain harboring the lastR gene. - E7, E9, and E11: Escherichia coli strains harboring neither the lastI gene nor the lastR gene

Figure 1B: M: Molecular weight marker, - CN: Negative control strain (**E. coli ATCC 25922)**
- E1 through E10: Strains of Pseudomonas aeruginosa and Escherichia coli in which the rhlI and rhlR genes were screened for.
- E1 and E3: Pseudomonas aeruginosa strains harboring the rhlI gene
- E1, E2, and E4: Pseudomonas aeruginosa strains harboring the rhlR gene. - E5: Pseudomonas aeruginosa strain harboring neither the rhlI gene nor the rhlR gene
- E8 and E9: Escherichia coli strains harboring the rhlI gene. – E9: Escherichia coli strain harboring the rhlR gene. – E6, E7, and E10: Escherichia coli strains harboring neither the rhlI gene nor the rhlR gene

Fig. 1. Electrophoretic profiles of the *LasI* and *LasR* genes (Figure 1A) and the *rhlI* and *rhlR* genes (Figure 1B) associated with quorum sensing in strains of *Escherichia coli* and *Pseudomonas aeruginosa*

Table 2. Prevalence of genes associated with quorum sensing in *Pseudomonas aeruginosa* and *Escherichia coli*

Quorum sensing genes	*P. aeruginosa* (n=12)	*E. coli* (n=7)	Total (n=19)
	Prevalence (%) and number of cases		
LasI	75,0 (9)	42,85 (3)	63,15 (12)
LasR	75,0 (9)	42,85 (3)	63,15 (12)
RhlI	58,33 (7)	71,42 (5)	63,15 (12)
RhlR	50,0 (6)	71,42 (5)	57,89 (11)

Las gene: Encodes the production of elastase; Rhl gene: Encodes the production of rhamnolipid resistant strains of *Pseudomonas aeruginosa* and *Escherichia coli*. All analyzed *P. aeruginosa* strains carried biofilm associated genes. The three biofilm forming genes (*ppy*R, *Pel*A and *Psl*A) were detected in some *Pseudomonas* strains, while others possessed two or only one of these genes. The biofilm forming genes *Psl*A, *Pel*A, and *ppy*R were detected in all twelve multidrug resistant *Pseudomonas aeruginosa* strains (Figure 2A). In the case of *Escherichia coli* strains, some carried the biofilm associated genes *Psl*A and/or *Pel*A, whereas others did not present any of them (Figure 2B).

Prevalences of *Psl*A, *Pel*A, and *ppy*R were respectively 50.0%, 41.66%, and 58.33%. In contrast, among the seven multidrug resistant *Escherichia coli* strains, only the biofilm forming genes *Psl*A and *Pel*A were identified, with prevalences of 28.57% and 14.28%, respectively. No gene encoding pyoverdine production (*ppy*R) was detected in *E. coli* (Table 3).

Distribution of biofilm forming genes in *Pseudomonas aeruginosa* and *Escherichia coli*

Principal component analysis (PCA) axes 1 and 2 together account for 100% of the total variance. Axis 1 of the PCA individual plot shows that the biofilm forming genes *Psl*A and *Pel*A are more closely associated with *Escherichia coli* strains than with *Pseudomonas aeruginosa* strains, which are the only ones harboring *ppy*R. However, axis 2 indicates that *P. aeruginosa* strains are more strongly associated with the genes *ppy*R and *Psl*A. (Figure 3).

DISCUSSION

In this investigation, isolates of *Escherichia coli* and *Pseudomonas aeruginosa* were found to contain genetic components involved in quorum sensing and biofilm development. The *Las* or *Rhl* genes were present in certain strains of *Escherichia coli* and *Pseudomonas aeruginosa*.

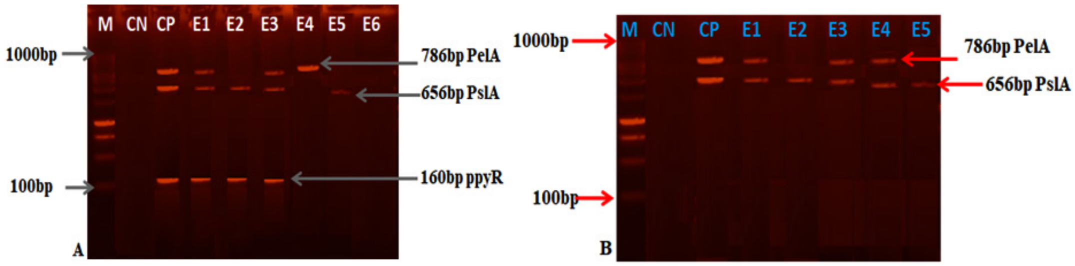
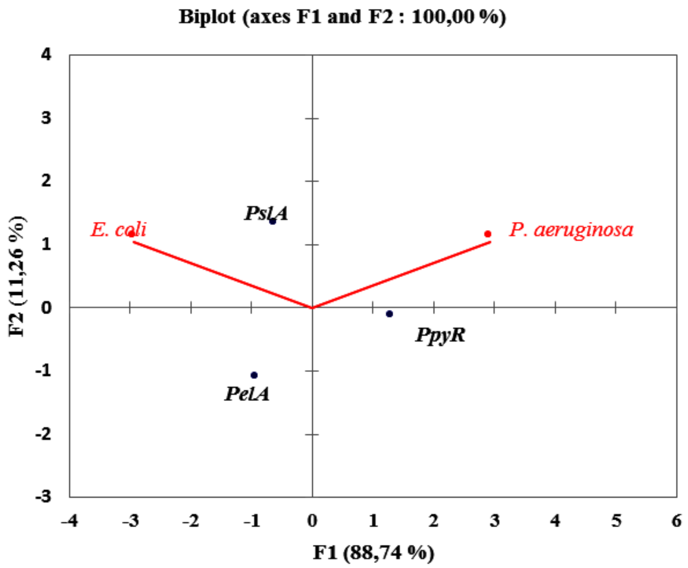


Fig. 2. Electrophoretic profiles of the genes *Psl*A, *Pel*A, and *ppy*R associated with biofilm in strains of *Escherichia coli* and *Pseudomonas aeruginosa*

Table 3. Prevalence of genes associated with Biofilm in *Pseudomonas aeruginosa* and *Escherichia coli*

Biofilms-associated genes	*P. aeruginosa* (n=12)	*E. coli* (n=7)
	Prevalence (%) and number of cases	
PslA	50,0% (6)	28,57% (2)
PelA	41,66 % (5)	14,28% (1)
PpyR	58,33% (7)	0,0% (0)
PslA-PelA	16,66% (2)	33,33% (5)
PslA-PpyR	8,33% (1)	0% (0)
PelA- PpyR	0% (0)	0% (0)
PslA-PelA-PpyR	25,0% (3)	0% (0)

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



PCA: Principal Component Analysis

Fig. 3. Distribution of genes involved in biofilm formation by bacterial strain

The Las gene encodes elastase production, whereas the *Rhl* gene encodes rhamnolipid production. These genes were detected in *P. aeruginosa* isolates with prevalences of 75.0% for *Las*I/R, 58.33% for *Rhl*I, and 50.0% for *Rhl*R. In *E. coli*, *Las* and *Rhl* genes were detected with prevalences of 42.85% (*Las*I/R) and 71.42% (*Rhl*I/R), respectively. The relatively high frequencies of these autoinducers in *P. aeruginosa* and *E. coli* strains may support the existence of a communication system between these bacteria isolated from animal products. *Quorum sensing* in these strains of *P. aeruginosa* and *E. coli* may be under the control of the *Las*I/R and *Rhl*I/R systems, which are involved in the synthesis of certain virulence genes.[28,29]

In Gram negative bacteria, two main QS effector/receptor systems involving homoserine lactones coexist and regulate the expression of virulence genes.[30] The 3 oxo C_{12} homoserine lactone are involved in the *Las*I/R system, which codes for virulence factors like elastases *Las*A and *Las*B and exotoxin A (ToxA). The second system, *Rhl*I/R, is responsible for the production and regulation of factors such as rhamnolipids, pyocyanin, and cyanide, and involves C4 homoserine lactone (C4 HSL).[31,32] The presence of *Las* and *Rhl* genes in both *Pseudomonas aeruginosa* and *Escherichia coli* isolates from smoked fish suggests that they may be involved in the production of bacterial pheromones that facilitate quorum sensing.

Nutrient limitation, stress, and cell density influence the biosynthesis of rhamnolipids.[33,34] In addition to the genes involved in quorum sensing, this study identified several genes encoding biofilm formation. Specifically, genes *ppyR, pslA*, and *pelA* associated to the biofilm were detected in *Pseudomonas aeruginosa* with respective prevalences of 50.0%, 41.66%, and 58.33%. The principal genes *ppyR*, *pslA* and *pelA* detected in *P. aeruginosa* strains suggest that these isolates are respectively implicated in the synthesis of pyoverdine, polysaccharide, and pellicle.

The production of pyoverdine, polysaccharide, and pellicle observed in these strains could promote mechanisms involved in the pathogenicity of *P. aeruginosa* through quorum sensing.[32,35] In *Escherichia coli*, the same genes *pelA* (14.28%) and *pslA* (28.57%), implicated in the biosynthesis of polysaccharide and pellicle, were detected; however, no gene encoding pyoverdine production was identified. The absence of the *ppyR* gene in *E. coli* accounts for the lack of pyoverdine production in this strain.

Principal component analysis (PCA)

confirmed that the biofilm forming genes *pslA* and *pelA* are more closely associated with *Escherichia coli* strains than with *Pseudomonas aeruginosa* strains, which are the only isolates harboring *ppyR*.

The presence of numerous resistance genes, together with evidence of *quorum sensing* and biofilm formation, underscores the need for reliable and appropriate food preservation procedures and techniques, particularly for products of animal origin, throughout the processing chain. Their occurrence in food, especially smoked fish, represents a major public health, and their epidemic potential.

CONCLUSION

This study demonstrated that *Pseudomonas aeruginosa* and *Escherichia coli* isolated from smoked fish sold in markets in Abidjan harbored quorum sensing and biofilm formation gene. *Quorum sensing* genes (*las*, *rhl*) and biofilm associated genes (*pslA*, *pelA*, *ppyR*) were identified in the isolates. No *ppyR* gene was detected in *E. coli*. The study also showed that all of these determinants may contribute to the virulence and pathogenicity of strains isolated from smoked fish. Improvements in smoking and preservation techniques for smoked fish are therefore required. Microorganisms found in smoked fish and the consumption of these products must be monitored. It is therefore recommended that vendors of smoked fish improve their work environment and adhere to hygiene standards regarding the workplace, equipment, and handlers, as well as proper packaging techniques for smoked fish. The presence of genes involved in biofilm formation and quorum sensing should serve as a warning to prevent public health issues.

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Conflict of interest

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Ethics Statement

This research did not involve human participants, animal subjects, or any material that requires ethical approval.

Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

Clinical Trial Registration

This research does not involve any clinical trials.

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Not Applicable.

Author Contributions

N'zebo Desire KOUAME: Conceptualization, Validation, Methodology, Writing - original draft, Writing - review & editing. Visualization, Formal analysis; Moumouny TRAORE: Analysis and interpretation of data, - writing the article; Benie Comoe Koffi Donatien: Investigation, Formal analysis; Atobla Koua: Investigation, - proofreading and editing the manuscript; Dadie Adjehi: Formal analysis, - final approval of the version to be published.

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