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BIOFILM PRODUCTION AND MULTIDRUG RESISTANCE IN BACTERIAL ISOLATES FROM VENDED MEAT-PIE IN OSOGBO, SOUTHWEST NIGERIA

Folasade M. ADEYEMI[1*], Andrew O. OLUWAYILEKA[2], Alimat O. USAMAT[1], Nana A. YUSUF-OMOLOYE[1], and Olaoniye H. AJIGBEWU[1].
Email: folasade.adeyemi@uniosun.edu.ng

Authors Affiliation:

[1]Department of Microbiology, Osun State University, Osogbo, Nigeria

[2]Department of Microbiology, University of Ibadan, Ibadan, Nigeria.

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ABSTRACT

Biofilms are microbial communities of surface-connected cells within an extracellular polymeric matrix that increases bacterial resistance to antimicrobials and external trauma, thereby enhancing their virulence. Ready-to-eat (RTE) foods have become popular and easily accessible; however, they pose serious health risks due to contamination at various stages of processing and supply. This study characterised bacterial species in meat pie fillings obtained from Osogbo, Nigeria, and determined their biofilm-forming and antibiotic-susceptibility patterns. A total of 40 meat pie samples were analysed using conventional microbiological techniques and the ABIS Online Bacterial Identification software. Biofilm production was determined using Congo red agar (CRA) and tissue culture plate (TCP) assays, and antibiotic sensitivity testing using the Kirby-Bauer disc diffusion technique. Approximately 95.0% of the samples yielded bacterial growth, comprising 45 isolates of 13 genera and 16 species, predominantly *Paenibacillus massiliensis* (44.4%). All isolates (100.0%) tested positive for biofilm production using the CRA, but only 46.7% with the TCP assay. The highest resistance rates were against amoxicillin, vancomycin, and erythromycin (100%). All isolates were multidrug-resistant with Multiple Antibiotic Resistance Index (MARI) values ≥ 0.4. Biofilm-forming, multidrug-resistant opportunistic pathogens, purportedly implicated in foodborne outbreaks in meat-pie fillings, pose a risk of severe and life-threatening infections in consumers. We therefore recommend strict compliance with food safety practices during meat pie pre- and post-production, vending and storage to minimize food safety hazards.

1. INTRODUCTION

Food provides nutrition, energy, and overall well-being, making it a basic human need for sustenance. Ready-to-eat (RTE) foods are intended for consumption at the point of purchase. They can be cooked or uncooked, hot or cold, and do not require additional heat treatment (Obande et al., 2018). Snacks are a category of RTE foods, and one of the most commonly consumed snacks in Nigeria is the meat pie, also known as Mincemeat pie. It is usually baked or sometimes fried, especially in Southwest Nigeria. It comprises a pastry crust filled with a mixture of spiced meats, diced potatoes, carrots, vegetables, stocks, and occasionally other animal products, such as boiled eggs (Mclauchlin et al., 2016). Examples include fruit, cream, custard, meat or chicken pies. Meat and other pies are well-liked globally due to their convenience for sale and consumption (Obande et al., 2018). However, meat pie is a food, and the processing surface becomes an ideal environment for biofilm formation when nutrients for microbial growth and attachment are sufficient, limiting its shelf life to 72–96 hours (Ezeh et al., 2017).

Despite its importance, there has been an increase in foodborne illness outbreaks due to risky food preparation and eating habits (Eke & Elechi, 2021). As a result of laxity in the enforcement of food safety practices by regulatory agencies, the hygiene levels and ethics of many food vendors are largely unsupervised, thereby compromising food quality, leading to the supply of potentially unsafe ready-to-eat foods to consumers. An estimated 200 disease types can be attributed to the consumption of contaminated foods (WHO, 2015), some of which are implicated in long-term health issues, particularly in the elderly and other vulnerable populations like toddlers and expectant mothers (Mengistu et al., 2022). Also, food and waterborne pathogens are the major causes of diseases in developing nations.

Bacterial biofilms are well-organized, complex communities of bacterial cells embedded in a self-synthesized extracellular matrix that adheres to inert or biologically derived surfaces (Flemming et al., 2016; Sharma et al., 2023, Ban-Cucerzan et al., 2025). Biofilm formation has been recognized as a significant factor in bacterial resistance and in the development of nosocomial infections (Sharma et al., 2023). This is because surface-adhered microbial groups can be found in diverse environments, including food, medical, industrial, and natural ecosystems. The presence of a biofilm in a bacterial infection indicates that the infection is therapy-resistant and that the likelihood of relapse is higher (Hardy et al., 2017; Sharma et al., 2023). Biofilm formation is a dynamic process: attachment, microcolony formation, biofilm maturation, and dispersion or detachment (Maunders & Welch, 2017; Abebe, 2020; Sharma et al., 2023). The biofilm matrix comprises extracellular polymeric substances (EPS), proteins, exogenous deoxyribonucleic acid (DNA), and lipids (Schilcher & Horswill, 2020; Rather et al., 2021; Chiba et al., 2022; Zhao et al., 2023). It also includes other components such as polysaccharides, ribonucleic acid (RNA) molecules, ions, and water. Biofilms impede host immunity and the effectiveness of antimicrobials (Høiby et al., 2010; Rather et al., 2021). Within bacterial biofilms, there is increased resistance to antibiotics, phagocytosis and opsonisation by antibodies, leading to chronic infections (Aslantaş & Demir, 2016; Guo et al., 2018; Hu et al., 2018; Ju et al., 2018).

The multiple antibiotic resistance index (MARI) is an endorsed, cost-effective, rapid, and user-friendly technique for tracking bacterial sources and prior antibiotic exposure (Krumperman, 1983; Mir, 2022). It is used to detect, characterise, and index isolates from high-risk environments and antibiotic pressure, with values ≥ 0.2 indicating high antibiotic-pressure sources. Prior reports have confirmed that biofilms are resistant to antibiotics and a myriad of disinfectants, highlighting the need to characterise and MAR index associated bacterial isolates as a crucial component of infection control (Høiby et al., 2010). Data on biofilm production by a bacterial isolate would assist clinicians in understanding its virulence and in formulating appropriate therapeutic options in the event of an infection or foodborne disease. Previous reports indicate that the Minimum Inhibitory Concentration (MIC) of antibiotics against

biofilms can be up to a thousand times greater than that for free forms of the same organism (Høiby et al., 2010). In addition, the impact of biofilm production in various areas of food production has been reported, leading to food spoilage, equipment damage, waste, disease outbreaks, and even fatalities (Giaouris & Simoes, 2018; Ban-Cucerzan et al., 2025). Pathogens can adhere to food-processing equipment, grow, and form biofilms, thereby increasing the risk to food safety (Hoveida et al., 2019). Hence, biofilm production is a marker of virulence in clinically relevant bacteria.

Given the growing interest in bacterial biofilm production in recent years, numerous studies on biofilm production in various bacterial species have been conducted. However, these studies primarily focus on device-associated biofilms (such as those associated with medical implants) and non-device-associated biofilms (found on tissues within the host, including skin, intestinal mucosa, oral cavity, and vagina) (Wi & Patel, 2018; Rather et al., 2021; Sharma et al., 2023; Zhao et al., 2023). There is a need to investigate biofilm production in bacteria from many other sources, including RTE foods. The objective of the present study was therefore to determine the biofilm production and antibiotic resistance profile of bacterial isolates recovered from RTE meat pies in Osogbo, southwestern Nigeria.

Materials and Methods

Study location, sample collection and processing

Forty (40) meat pie samples (19 from selected shops/eateries/roadside sellers and 21 from mobile vendors) were collected across Osogbo, Osun State, Nigeria. The samples were collected in sterile Ziplock pouches (250 ml), labelled appropriately, stored in cool boxes with ice packs, and then transferred to the Microbiology Laboratory of Osun State University, Osogbo, Nigeria, within 4 hours after collection for immediate microbiological analysis.

One (1) gram of each meat pie filling was aseptically measured into 9 ml of sterile Ringer solution in labelled test tubes and homogenised by vigorous shaking for one minute each using a vortex mixer. One (1) ml of this suspension was then inoculated into 9 ml of sterile Tryptone Soy broth (TSB) and incubated at 37±2 ℃ for 18 - 24 hours. Subsequently, the broth cultures were inoculated by streaking onto Tryptone Soy Agar (TSA) and MacConkey agar plates, and incubated for an additional 24 hours at 37±2 ℃ to obtain pure isolates. Distinct colonies were observed and Gram-stained, then characterised by their morphological and cultural characteristics. Conventional biochemical analyses (catalase, oxidase, coagulase, deoxyribonuclease, haemolysis, motility, indole, citrate, methyl red, Voges-Proskauer and sugar fermentation [using mannitol, fructose, lactose, sucrose and glucose sugars]) and observed results were interpreted using the ABIS online Microbiology software for bacterial identification to identify the recovered isolates (ABIS online - bacterial identification software version 12, http://www.tgw1916.net).

Phenotypic screening for biofilm production

The biofilm-forming capability of the recovered bacterial isolates was determined using two methods: Growth on Congo Red Agar (CRA) and Tissue Culture Plate Assay.

Growth on Congo Red Agar (CRA) - This test was performed as previously described (Freeman et al., 1989; de Castro Melo P et al., 2013). The medium was composed of brain heart infusion broth (BHIB), sucrose (50 g/L), agar No. 1 (10 g/L), and Congo red (0.8 g/L). Congo red stain was prepared as a concentrated solution and sterilized by autoclaving at 121 ℃ for 15 minutes at 15 psi, separate from the rest of the medium components. It was supplemented to the agar upon cooling to 55 °C. Sterile CRA plates were inoculated and incubated aerobically for 18-24 hours at 37 ± 2 ℃. Black colonies with a dry, crystalline consistency indicated biofilm production, while colonies of non-biofilm-producing bacterial strains appeared red.

Tissue culture plate assay (TCP): The TCP assay described by Saber et al. (2022) was used, with slight modifications. Isolates from an overnight culture of TSA were inoculated into Brain Heart Infusion Broth (BHIB), incubated for 18-24 hours at 37±2 ℃, and then diluted 1:100 with fresh medium (by adding 0.1 mL of inoculum into 9.9 mL of sterile BHIB in a test tube). Individual wells of sterile, 96-well flat-bottom polystyrene tissue culture plates (Sigma-Aldrich, USA) were inoculated with 200 µL aliquots of freshly diluted cultures and incubated for 18-24 hours at 37 ± 2 °C. Uninoculated BHIB served as a negative control. The contents of each well were removed by lightly tapping the plates, upside down, onto a tissue pad to absorb excess moisture, and the wells were washed manually five times with 200 µL of phosphate-buffered saline (PBS) (pH 7.2). Biofilms in each well were fixed with methanol, and the wells were drained. They were then stained with 0.1% crystal violet (0.1% w/v) for 20 minutes, drained again, and washed off with distilled water. Finally, 250 µl of 33% glacial acetic acid was added to each well. The optical density (OD) of each well was measured (wavelength 570 nm) with an ELISA auto reader (Thermo Scientific). The test was done in triplicate for all isolates, and the average OD values were estimated. The results were interpreted as previously described, optical density cut-off value (ODc) being equivalent to the sum of the average OD of negative control and the 3x standard deviation (SD) of negative control (Table 1) (Stepanović et al., 2007).

Antibiotic Susceptibility profiling of recovered isolates

Antibiotic susceptibility testing (AST) was performed using the Kirby-Bauer disc diffusion method (Adeyemi et al., 2022), using 13 commercially available antibiotics from 11 different classes vis-aminoglycosides [amikacin (30 µg) and gentamicin (10 µg)]; β-lactam antibiotic/penicillin [amoxicillin (10 µg)]; β -lactam/inhibitor combination [amoxicillin clavulanic acid (20/10 µg)]; carbapenem [imipenem (10 µg)]; cephalosporin [cefotaxime (5 µg)]; fluoroquinolone [ciprofloxacin (5 µg)]; folate pathway antagonist [trimethoprim-sulfamethoxazole (1.25/23.75 µg)]; glycopeptide [vancomycin (5 µg)];

macrolides [erythromycin (15 µg)]; monobactam [aztreonam (30 µg)]; tetracycline [tetracycline (30 µg) and doxycycline (20 µg)]. The results were interpreted using the EUCAST breakpoint table (version 13.0) (EUCAST, 2023), while multidrug resistance (MDR) was defined as the resistance of an isolate to at least one drug in a minimum of three antibiotic classes. The multiple antibiotic resistance index (MARI) was evaluated using the equation below (Krumperman, 1983):

$$MARI = \frac{\text{Number of antibiotics to which the organism is resistant}}{\text{total number of antibiotics to which the organism was exposed}}$$

Results

Altogether, 40 meat pie fillings (observed to comprise a blend of meat, carrot and potato) were analysed. A total of 38 of the 40 samples (95.0%) yielded bacterial growth; only two samples had no growth. The frequency of recovered bacterial isolates from the 40 samples is shown in Figure 1. These include 45 bacterial isolates comprising 36 Gram-positive bacteria (80.0%) in 6 genera (3 phyla - Bacillota, Firmicutes and Actinomycetota and five families Paenibacillaceae, Bacillaceae, Lachnospiraceae, Listeriaceae and Micrococcaceae) - and 9 Gram-negative bacteria in 7 genera (a single phylum Pseudomonadota but 3 families - Erwiniaceae, Enterobacteriaceae and Pseudomonadaceae). *Paenibacillus massiliensis* was the predominant bacterial species at 44.4% (n = 20/45), followed by *Paenibacillus sanguinis* and *Bacillus megaterium*, each at 8.9% (n = 4/45). All 45 recovered bacterial isolates were obtained from the 38 samples – 32 samples (84.2%) had single isolates, whereas 6 samples (15.8%) were polymicrobial (five samples had 2 isolates each, while the last sample had three isolates, namely *Citrobacter rodentium, Pantoea agglomerans* and *Paenibacillus sanguinis*). The similarity indices for the identification using ABIS online software were ≥ 97% for all isolates.

Table 1. Interpretation of biofilm production

Average OD value	Values	Biofilm production
≤ ODc	≤ 0.254	None (-)
ODc < ~ ≤ 2x ODc	0.254 < x ≤ 0.507	Weakly positive (+)
2x ODc < ~ ≤ 4x ODc	0.507 < x ≤ 1.014	Moderately positive (++)
> 4x ODc	> 1.014	Strongly positive (+++)

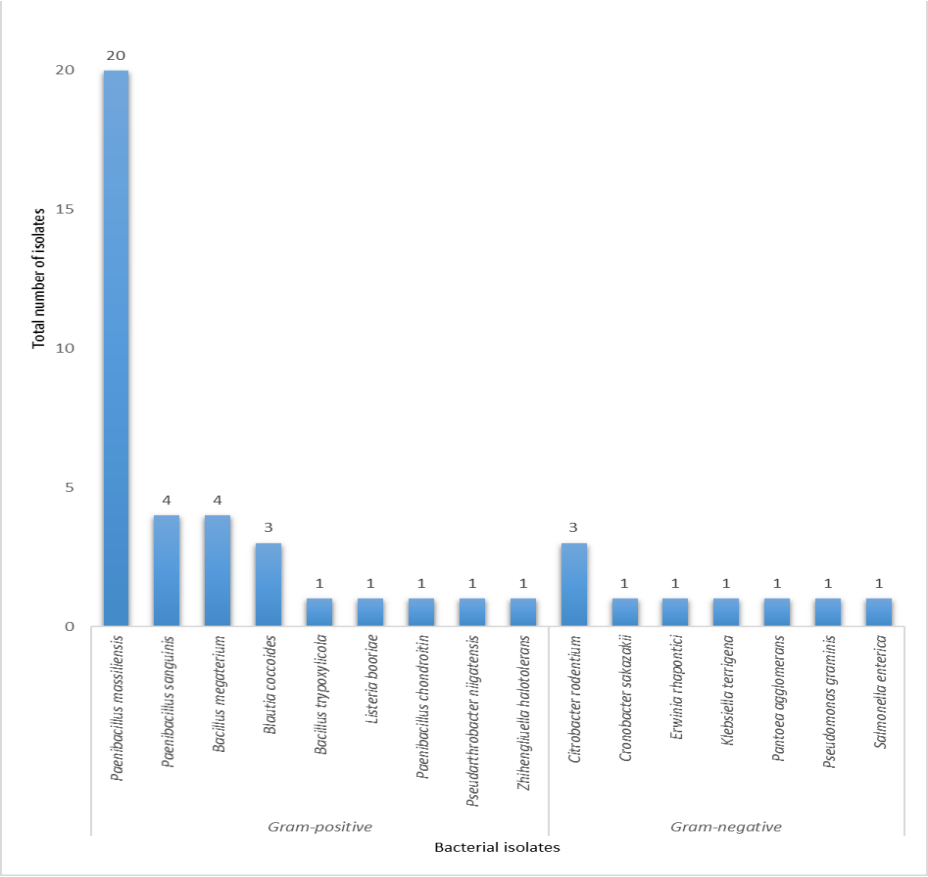

Figure 1. Frequency of bacterial isolates recovered from fillings of meat pie samples

Phenotypic screening for biofilm production

All 45 recovered isolates tested positive (i.e., exhibited black colonies with a dry, crystalline consistency) for biofilm production on the Congo red agar. The total number of biofilm producers which gave a positive result with the tissue culture plate assay was 21 in number (46.7%), comprising 16 Gram-positive (10 *Paenibacillus massiliensis,* 2 *Bacillus megaterium,* and 1 each of *Bacillus trypoxylicola, Blautia coccoides, Pseudarthrobacter niigatensis,* and *Zhihengliuella halotolerans*) and 5 Gram-negative isolates (2 *Citrobacter rodentium,* and 1 each of *Cronobacter sakazakii, Pantoea agglomerans,* and *Salmonella enterica*) produced biofilm. Five (23.8%), nine (42.9%) and 7 (33.3%) of the biofilm producers were strong, moderate and weak producers, respectively (Table 2).

The number of non-biofilm-producing isolates with the tissue culture plate assay was 24 (53.3%), made up of 20 Gram-positive (10 *Paenibacillus massiliensis,* 4 *Paenibacillus sanguinis,* 2 each of *Bacillus megaterium* and *Blautia coccoides*; and 1 each of *Listeria booriae* and *Paenibacillus chondroitin*); and 4 Gram-negative isolates (*Citrobacter rodentium, Erwinia rhapontici, Klebsiella terrigena* and *Pseudomonas graminis*).

Table 2. Screening for biofilm formation by Congo Red Agar and Tissue Culture Plate methods

BACTERIAL ISOLATE		Total tested	Biofilm Production Assay				
			CRA	Tissue Culture Plates Assay (TCP)			
				None	Weak	Moderate	Strong
Gram-positive	*Paenibacillus massiliensis*	20	20	10	3	4	3
	Paenibacillus sanguinis	4	4	4	0	0	0
	Bacillus megaterium	4	4	2	0	0	2
	Blautia coccoides	3	3	2	0	1	0
	Bacillus trypoxylicola	1	1	0	0	1	0
	Listeria booriae	1	1	1	0	0	0
	Paenibacillus chondroitin	1	1	1	0	0	0
	Pseudarthrobacter niigatensis	1	1	0	1	0	0
	Zhihengliuella halotolerans	1	1	0	0	1	0
Sub-Total		**36**	**36**	**20**	**4**	**7**	**5**
Gram-negative	*Citrobacter rodentium*	3	3	1	1	1	0
	Cronobacter sakazakii	1	1	0	1	0	0
	Erwinia rhapontici	1	1	1	0	0	0
	Klebsiella terrigena	1	1	1	0	0	0
	Pantoea agglomerans	1	1	0	0	1	0
	Pseudomonas graminis	1	1	1	0	0	0
	Salmonella enterica	1	1	0	1	0	0
Sub-Total		**9**	**9**	**4**	**3**	**2**	**0**
GRAND TOTAL		**45**	**45**	**24**	**7**	**9**	**5**

Antibiotic susceptibility profiling of recovered isolates

The results of the AST profile revealed that resistance was highest to amoxicillin, vancomycin, and erythromycin (100%), followed closely by imipenem and tetracycline at 89.7% and 88.6%, respectively. Low-level resistance was observed to aztreonam (10.0%) and amikacin (21.4%). Among the isolates tested for ciprofloxacin, gentamicin, and cefotaxime, no resistance was recorded (0.0%). Details of the antibiotic resistance profile of the recovered isolates are shown in Table 3. All isolates were multidrug-resistant, as they were resistant to ≥4 and ≤8 classes of antibiotics (Table 4). Additionally, all isolates (100.0%) had MARI values ≥ 0.4 (Figure 2).

Table 3. The antibiotic Resistance Profile of recovered bacterial isolates from vended meat pie samples

BACTERIAL ISOLATES		No Tested	Total number of resistant isolates (%)												
			AMK	GEN	AMX	AUG	IMP	CTX	CIP	SXT	VAN	ERY	ATM	TET	DXY
Gram-positive	*Paenibacillus massiliensis*	20	NT	NT	20	13	20	NT	0	10	20	20	NT	16	5
	Paenibacillus sanguinis	4	NT	NT	4	3	4	NT	0	2	4	4	NT	3	2
	Bacillus megaterium	4	NT	NT	4	2	4	NT	0	2	4	4	NT	4	1
	Blautia coccoides	3	NT	NT	3	2	NT	NT	NT	NT	3	3	NT	3	1
	Bacillus trypoxylicola	1	NT	NT	1	1	NT	NT	NT	NT	1	1	NT	1	0
	Listeria booriae	1	NT	NT	1	1	NT	NT	NT	NT	1	1	NT	1	NT
	Paenibacillus chondroitin	1	NT	NT	1	1	1	NT	0	1	1	1	NT	1	1
	Pseudarthrobacter niigatensis	1	NT	NT	1	NT	NT	NT	NT	1	1	1	NT	NT	NT
	Zhihengliuella halotolerans	1	0	NT	1	1	0	0	0	1	1	1	0	1	0
Gram-negative	*Citrobacter rodentium*	3	0	0	3	3	2	0	0	1	3	3	1	3	3
	Cronobacter sakazakii	1	0	0	1	1	1	0	0	0	1	1	0	1	1
	Erwinia rhapontici	1	1	0	1	1	1	0	0	1	1	1	0	1	1
	Klebsiella terrigena	1	0	0	1	1	0	0	0	1	1	1	0	1	1
	Pantoea agglomerans	1	0	0	1	1	0	0	0	0	1	1	0	1	0
	Pseudomonas graminis	1	1	NT	1	1	1	0	0	NT	1	NT	0	1	NT
	Salmonella enterica	1	0	0	1	1	1	0	0	0	1	1	0	1	1
TOTAL		45	2 (20.0)	0 (0.0)	45 (100.0)	33 (75.0)	35 (89.7)	0 (0.0)	0 (0.0)	20 (51.3)	45 (100.0)	44 (100.0)	1 (10.0)	39 (88.6)	17 (40.5)

Legend: AMK – amikacin; GEN – gentamicin; AMX – amoxicillin; AUG - amoxicillin clavulanic acid; IMP – imipenem; CTX – cefotaxime; CIP – ciprofloxacin; SXT - trimethoprim-sulfamethoxazole; VAN – vancomycin; ERY – erythromycin; ATM – aztreonam; TET – tetracycline; DXY – doxycycline; NT – Not tested.

Table 4. The Multiple Antibiotic Resistance pattern of bacterial isolates from vended meat pie samples

BACTERIAL ISOLATES		No Tested	Number of antibiotic classes against which isolates are resistant											
			0	1	2	3	4	5	6	7	8	9	10	11
Gram-positive	*Paenibacillus massiliensis*	20	0	0	0	0	0	6	9	5	0	NT	NT	NT
	Paenibacillus sanguinis	4	0	0	0	0	0	1	2	1	0	NT	NT	NT
	Bacillus megaterium	4	0	0	0	0	0	0	4	0	0	NT	NT	NT
	Blautia coccoides	3	0	0	0	0	1	2	NT	NT	NT	NT	NT	NT
	Bacillus trypoxylicola	1	0	0	0	0	0	1	NT	NT	NT	NT	NT	NT
	Listeria booriae	1	0	0	0	0	0	1	NT	NT	NT	NT	NT	NT
	Paenibacillus chondroitin	1	0	0	0	0	0	0	0	1	0	NT	NT	NT
	Pseudarthrobacter niigatensis	1	0	0	0	0	1	NT	NT	NT	NT	NT	NT	NT
	Zhihengliuella halotolerans	1	0	0	0	0	0	0	1	0	0	0	0	0
Gram-negative	*Citrobacter rodentium*	3	0	0	0	0	0	0	2	1	0	0	0	0
	Cronobacter sakazakii	1	0	0	0	0	0	0	1	0	0	0	0	0
	Erwinia rhapontici	1	0	0	0	0	0	0	0	0	1	0	0	0
	Klebsiella terrigena	1	0	0	0	0	0	0	1	0	0	0	0	0
	Pantoea agglomerans	1	0	0	0	0	0	1	0	0	0	0	0	0
	Pseudomonas graminis	1	0	0	0	0	0	0	1	0	0	0	0	0
	Salmonella enterica	1	0	0	0	0	0	0	1	0	0	0	0	0
TOTAL		45	0	0	0	0	2	12	22	8	1	0	0	0

Legend: NT – Not tested

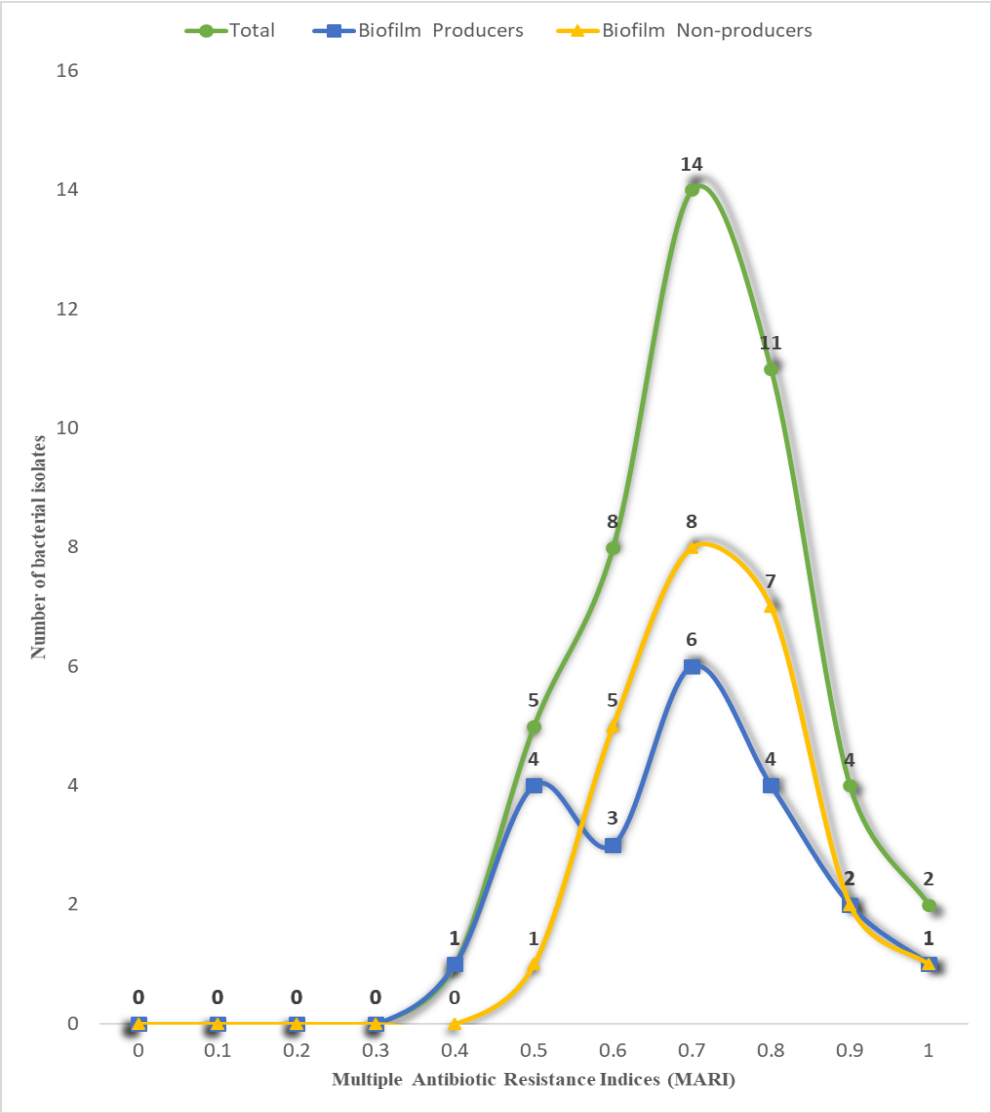

Figure 2. The Multiple Antibiotic Resistance Indices of biofilm and non-biofilm-producing isolates recovered from vended meat pie

Antibiotic resistance profile of biofilm and non-biofilm producers

For the biofilm-producing isolates, the highest resistance was exhibited against amoxicillin, vancomycin and erythromycin, closely followed by tetracycline. The highest level of susceptibility was observed against cefotaxime, ciprofloxacin, and gentamicin. The same trend was observed in the non-biofilm producers, with both groups exhibiting similar patterns of resistance and sensitivity (Table 5).

The lowest MARI value for biofilm-producing isolates was 0.4, and for non-producers, 0.5. Also, for the biofilm producers, the MARI value with the highest number of isolates was 0.7; ditto for the non-biofilm producers (Figure 2).

Table 5. Resistance pattern (%) of biofilm-producing and non-biofilm-producing bacterial isolates

BACTERIAL ISOLATES		No Tested	Total number of resistant isolates											
			AMK	GEN	AMX	AUG	IMP	CTX	CIP	SXT	VAN	ERY	ATM	TET	DXY
Biofilm	Gram-positive	16	NT	NT	16	10	12	NT	0	10	16	16	NT	14	2
Producers	Gram-negative	5	0	0	5	5	3	0	0	1	5	5	0	5	4
(n=21)	Total	21	0	0	21	15	15	0	0	11	21	21	0	19	6
Non-Biofilm	Gram-positive	20	0	0	20	14	17	0	0	7	20	20	0	16	8
Producers	Gram-negative	4	2	0	4	4	3	0	0	2	4	3	1	4	3
(24)	Total	24	2	0	24	18	20	0	0	9	24	23	1	20	11
GRAND TOTAL		45	2	0	45	33	35	0	0	20	45	44	1	39	17

Legend: AMK – amikacin; GEN – gentamicin; AMX – amoxicillin; AUG - amoxicillin clavulanic acid; IMP – imipenem; CTX – cefotaxime; CIP – ciprofloxacin; SXT - trimethoprim-sulfamethoxazole; VAN – vancomycin; ERY – erythromycin; ATM – aztreonam; TET – tetracycline; DXY – doxycycline; NT – Not tested

Discussion

This study isolated and characterised 45 bacterial isolates from 40 meat pie fillings, screened for biofilm production and determined the antibiotic susceptibility patterns of the recovered isolates. The single predominant bacterial species recovered was *Paenibacillus massiliensis* (at the rate of 44.4%). The genus *Paenibacillus* comprises Gram-positive, or sometimes Gram-variable, spore-forming rods (Sáez-Nieto et al., 2017), formerly classified within the genus *Bacillus* (Ash et al., 1993; Patowary & Deka, 2020). They are especially versatile and resilient with characteristic features that enable them to survive in a wide range of habitats, diverse environments and dissimilar samples (Grady et al., 2016; Tonial et al., 2020). They have been isolated from various sources, including but not limited to human clinical samples (Sáez-Nieto et al., 2017; Tonial et al., 2020; Roux & Raoult, 2004), the soil, spring water, human faecal matter, insect larvae (Xu et al., 2017) and even food samples (Berge et al., 2002), including milk, both in the pasteurized and unpasteurized form (Beno et al., 2020). The genus *Paenibacillus* has been reported to cause spoilage of bakery products and pastries (Valerio et al., 2012). They are known to produce many spoilage enzymes, and as spore producers, they are typically resistant to extremes of temperature, which may be the case during baking at high temperatures (André et al., 2017).

Paenibacillus spp. are not typically implicated in human infections or disease causation and are generally not harmful to their hosts. However, they are opportunistic pathogens as they have been reported to be associated with various infections in immunosuppressed individuals (Grady et al., 2016). Despite the challenges in differentiating between true pathogens and contaminating isolates, a previous study reported that approximately 25% of their isolates were implicated in actual human infections, as the authors established relationships with bacteraemia (Sáez-Nieto et al., 2017). Therefore, the presence of *Paenibacillus* spp. in RTE food samples

in the present study is concerning and could potentially lead to foodborne diseases in consumers. Of the *Paenibacillus* spp., only *P. massiliensis* produced biofilms, with 10 isolates (50.0%) of the recovered *Paenibacillus massiliensis* isolates in this study being biofilm producers with the TCP method (3 isolates each were weak and strong producers, while 4 were moderate biofilm producers). However, all isolates were positive biofilm producers with the CRA assay. The findings of many previous studies contrast with trends in detection rate between the CRA and TCP methods (Harika et al., 2020; Singh and Chand, 2025) in this study.

The disparity between the detection rates of the two methods in this study is not strongly apparent, but it could be due to the constitution of the media, as the addition/concentration of sugar plays a significant role in biofilm production (Jha et al., 2022; Plotkin et al., 2025). In this study, sucrose (50 g/L [5%]) was added to the CRA constituted for the assay. False positive results have also been reported with the CRA method, but not with the TCP method (Harika et al., 2020). Singh and Chand (2025) also observed that discrepancies observed in the CRA method in various studies might be because different studies used modifications in the media with varying sugar content, and that duration of incubation impacted biofilm production by the bacteria.

Resistance to antibiotics was high in all the *Paenibacillus* strains tested. Absolute resistance to the beta-lactams, carbapenems, vancomycin and erythromycin was recorded. This was in line with the study of Sáez-Nieto et al. (2017), where 95.6% of their *Paenibacillus* isolates were also resistant to ampicillin. However, the same authors reported lower resistance rates to erythromycin and vancomycin at 13% and 30%, respectively. Variations in the resistance patterns in *Paenibacillus* strains have been established to be strain-dependent (Grady et al., 2016), and this possibility increases with the ability of the different strains to have varying biofilm-producing capabilities, as shown in the present study.

For the Gram-negative isolates, *Citrobacter rodentium* was the principal organism recovered. Although it is affiliated with rodents, it can cause opportunistic infections in humans. Other bacterial species were all members of the family *Enterobacteriaceae* except for *Pseudomonas graminis,* with the majority of the members being foremost foodborne bacterial pathogens that have been found associated with food-related outbreaks. Sources of the foods affected range widely from traditional foods like beef and dairy to non-conventional foods like spices, condiments, RTE foods, dough, nuts and dried flour (Janda & Abbott, 2021).

Cronobacter sakazakii is an emerging opportunistic foodborne pathogen previously implicated in human infections, including conjunctivitis, pneumonia, diarrhoea, urinary tract infections, abscesses, and wounds in adults (Yong et al., 2018; Mazi et al., 2023a). It has been recovered from various food types, including RTE foods (Pakbin et al., 2022; Pakbin et al., 2023) and was reported to be responsible for a gastroenteritis outbreak in students and employees of a high school in China

(Yong et al., 2018).

The resistance pattern of *Cronobacter sakazakii* to erythromycin and amoxicillin was consistent with the study by Yong et al. (2018). The isolate in the present study was also sensitive to cefotaxime, gentamicin, ciprofloxacin, and trimethoprim/sulfamethoxazole as reported by Yong et al. (2018). In another study (Pakbin et al., 2022), *Cronobacter sakazakii* isolates were resistant to amoxicillin, amoxicillin-clavulanic acid, and erythromycin, and susceptible to gentamicin, which is consistent with the single isolate in our study. Nevertheless, the resistance patterns to ciprofloxacin and tetracycline varied from those in our study.

The presence of multidrug-resistant opportunistic pathogenic bacteria in RTE meat pies, some with spore-forming and biofilm-producing abilities as recorded in this study and the consumption of RTE pastry contaminated with such isolates signal possible public health hazards. Within the Osogbo township, many such meat pies are purchased from eateries, fast-food restaurants, roadside vendors, and even mobile vendors. Approximately half of the samples (52.5%; 21/40) in this study were obtained from mobile vendors. The practice of purchasing fast and RTE foods has become rampant due to increasingly busy daily schedules in big towns like Osogbo, work demands, extensive working hours, extended school hours for school-aged children and commuting from place to place. Therefore, RTE foods are very convenient as they are easily available and mostly reasonably priced. This habit, however, constitutes a risk, as a recent study evaluating the food safety practices of consumers and food vendors in Nigeria reported that observed food safety practices of food vendors are worse than their safety knowledge or self-reported practices (Nordhagen, 2022). In a review by Mazi et al. (2023b), it was surmised that several factors among street food vendors in Nigeria capable of contributing to the burden of foodborne diseases included unhygienic practices and poor handling and preparation, improper storage of leftovers, lack of access to clean water and a lack of proper food safety training.

Conclusion

This study investigated the biofilm-producing capabilities and antibiotic resistance patterns of bacterial isolates recovered from meat pie samples in Osogbo, Southwestern Nigeria. Our studies show that the TCP assay was more discriminatory than the Congo Red agar method for assessing biofilm production, and the recovered isolates were opportunistic pathogens capable of causing food spoilage or implicated in foodborne infections. The predominant organism recovered was *Paenibacillus massiliensis,* while 46.7% of the isolates were biofilm producers as determined by the TCP assay. All recovered isolates were multidrug-resistant and had MARI values ≥ 0.4, indicating that they originated from an environment under antibiotic pressure. However, a key limitation of this study is the inability to characterise our isolates using molecular methods and to screen for biofilm and antibiotic-resistance genes due to financial constraints.

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Data Availability: All data included: All the data supporting the results of this study are included in the article itself.

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