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Biofilm Formation Capabilities of Lactobacillus Species Isolated from Selected Fermented Food Products Using a Statistical Approach

Blessing Adoh Olodu[1], Stephen Amadin Enabulele[1]

[1] Department of Biological Science (Microbiology), Benson Idahosa University, Benin City, Edo State, Nigeria

ABSTRACT

Background: This study investigates the biofilm formation capabilities of Lactobacillus species isolated from fermented cassava and corn products. Understanding biofilm formation is crucial for evaluating the probiotic potential of these species, as biofilm-forming ability influences their survival and functionality in host environments.

Methods: Nine bacterial isolates, including Lactobacillus fermentum, L. ghanensis, L. delbrueckii, L. plantarum, Lactococcus lactis, L. reuteri, Lysinibacillus sphaericus, Bacillus cereus, and B. pacificus, were assessed for biofilm production using the microtiter plate assay. After crystal violet staining, optical density (OD) values were measured at 570 nm spectrophotometrically. Based on OD values, isolates were classified into four categories: no biofilm, weak, moderate, and strong biofilm formation. Statistical analyses, including two-stage least squares regression, were employed to evaluate biofilm formation trends and predictors.

Results: The predictive regression model was highly significant ($R^2 = 0.987$, $F = 122.618$, $p < 0.0001$). Biofilm formation strength varied, with the highest mean percentage observed in the moderate group (31.29%), followed by weak (27.41%), strong (20.46%), and no biofilm (20.05%). Among the isolates, Lactobacillus fermentum exhibited the highest rate of strong biofilm formation (46.1%), while Lysinibacillus sphaericus showed none. Moreover, The highest biofilm formation was observed at 37°C (31.29%), followed by 25°C (27.41%), and 45°C (20.46%). Similarly, biofilm formation was highest at pH 6.5 (30.41%), followed by pH 7.5 (25.39%) and pH 4.5 (20.05%). Lactobacillus fermentum exhibited the highest strong biofilm formation (46.1%) at 37°C and pH 6.5.

Conclusion: Biofilm formation in Lactobacillus species is species-specific and environmentally influenced by temperature and pH. Lactobacillus fermentum demonstrated strong biofilm formation, making it a promising candidate for probiotic applications.

Keywords: Biofilms formation, crystal violet staining, Fermentation, Lactobacillus species, Probiotics, Statistical models

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Address for correspondence: *Blessing Adoh Olodu , Department of Biological Science (Microbiology), Benson Idahosa University, Benin City, Edo State, Nigeria; E-mail: blessingolodu18@gmail.com*

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The study of biofilm formation capabilities of Lactobacillus species has several clinical benefits. Lactobacillus species with strong biofilm-forming abilities are more likely to adhere to the intestinal epithelium, resist gastric acids, and survive bile salts, enhancing their colonization potential and making them effective probiotics for gut health.[1, 2] Biofilms provide a protective matrix that shields bacteria from hostile environments, ensuring sustained delivery of health benefits. These probiotics can modulate gut microbiota, helping prevent or treat conditions such as irritable bowel syndrome (IBS), inflammatory bowel disease (IBD), and diarrhea.[2, 3] Additionally, their biofilm-forming ability aids in the competitive exclusion of pathogens by occupying niches on the gut lining. Probiotic Lactobacillus species with antimicrobial properties can help manage infections caused by harmful or antibiotic-resistant bacteria.[2] They may also reduce the risk of dental caries and periodontal diseases by preventing pathogenic biofilms in the oral cavity. Variability in biofilm formation across species provides insights for tailoring probiotic supplements to individual health needs, facilitating personalized dietary interventions for improving gastrointestinal health or preventing specific conditions. Demonstrating that fermented foods contain biofilm-capable Lactobacillus species supports their role as functional foods with preventive health benefits beyond basic nutrition.[3, 4] Moreover, Lactobacillus biofilms could be engineered for drug delivery, offering sustained release of therapeutic agents, particularly in gastrointestinal treatments. This foundational knowledge contributes to advancing probiotic therapy, infection management, functional food development, and innovative drug delivery systems, significantly impacting public health.

Fermented foods have been central to traditional diets worldwide, offering not only unique flavors but also significant health benefits. Notably, fermented cassava and corn products are integral to African cuisine, particularly in Nigeria, where they are staple components. The fermentation process promotes the growth of beneficial microorganisms, primarily lactic acid bacteria (LAB), which are essential for food preservation and improving nutritional value.5,6 Increasing attention has been given to LAB, particularly Lactobacillus species, because of their well-documented probiotic properties, which include gut health support, immune modulation, and inhibition of pathogenic bacteria.[7, 8]

Probiotics are defined as live microorganisms that provide health benefits to the host when administered in sufficient quantities.[9, 10] Lactobacillus species have been widely studied for their resilience to acidic and bile conditions, adherence to the intestinal mucosa, and production of antimicrobial substances like bacteriocins.[11, 12] The rising demand for natural, functional foods fortified with probiotics has fueled research efforts to isolate and characterize promising probiotic strains from traditional fermented foods.[13, 14] The potential health benefits of Lactobacillus species include preventive measures against gastrointestinal infections and chronic illnesses.[15, 16]

Despite their widespread use, research on the probiotic potential of LAB from fermented cassava and corn in Nigeria remains limited. Previous studies have primarily focused on dairy-based fermented products, while non-dairy sources, which are often more accessible in tropical regions, have been underexplored.[17, 18] The ability of these isolates to form biofilms is crucial, as biofilm formation enhances bacterial adherence to the intestinal lining, potentially improving gut colonization and probiotic effectiveness.[19, 20] Biofilms, as complex microbial communities, offer protection against environmental stressors, potentially increasing the bacteria's survival and functionality within the gastrointestinal tract.[21, 22] This research also assess biofilm formation as an indicator of efficient gut colonization. By advancing the understanding of indigenous probiotic strains, the study aims to contribute to the development of functional foods and therapeutic strategies that can address prevalent health challenges in Nigeria and beyond.[23, 24] Furthermore, the results could support the creation of locally produced, sustainable probiotic products, enhancing health and nutritional security.[25, 26] Biofilm formation is a critical characteristic of Lactobacillus species, significantly influencing their probiotic functionality and resilience in various environments. Factors like genetic diversity and stress conditions can impact biofilm strength, which varies among species and samples.[27-29] Understanding these differences aids in optimizing probiotic applications.[30-34]

METHODS

This research focused on the isolation, identification, and biofilm formation assessment of Lactobacillus species from fermented cassava and corn samples. A systematic approach was adopted, including sample

collection, microbial isolation, biofilm formation assessment, and statistical analysis.

Sample Collection

Fermented cassava and corn samples were collected from various local markets in Benin City, Nigeria. Samples were transported in sterile containers to the laboratory and processed within 24 hours to ensure the viability of the microorganisms.[3]

Microbial Isolation

The isolation of Lactobacillus species was performed using serial dilution and plating techniques. A 10 g sample of each fermented product was homogenized in 90 mL of sterile peptone water and serially diluted up to 10^{-6}. Aliquots (0.1 mL) of the appropriate dilutions were spread on de Man, Rogosa, and Sharpe (MRS) agar plates. Plates were incubated at 37°C for 48 hours under anaerobic conditions using an anaerobic jar with gas-generating kits. Colonies displaying typical Lactobacillus morphology (smooth, round, and cream-colored) were selected and purified by sub-culturing.

Assessment of Biofilm Formation

The biofilm-forming ability of the Lactobacillus isolates was evaluated using the microtiter plate assay. Overnight cultures of each isolate were adjusted to an optical density of 0.5 at 600 nm, corresponding to approximately 10^8 CFU/mL. A 200 µL aliquot of each culture was transferred into wells of a sterile, flat-bottomed 96-well polystyrene microtiter plate. The wells were incubated at 37°C for 24 hours under anaerobic conditions.[20, 21] After incubation, wells were washed three times with phosphate-buffered saline (PBS) to remove non-adherent cells. Adherent biofilms were fixed with 99% methanol for 15 minutes and stained with 0.1% crystal violet for 20 minutes. Excess stain was rinsed off with distilled water, and the plates were air-dried. The bound crystal violet was solubilized with 33% acetic acid, and the absorbance was measured at 570 nm using a microplate reader.[15,20]

Categorization of Biofilm Formation

The strength of biofilm formation was categorized based on the absorbance values: no biofilm (OD ≤ 0.1), weak (0.1 < OD ≤ 0.2), moderate (0.2 < OD ≤ 0.4), and strong (OD > 0.4). The experiment was performed in triplicate for each isolate, and the mean absorbance values were calculated.

Statistical Analysis

All assays were conducted in triplicate to ensure data reliability. Statistical analyses were performed using appropriate software, such as SPSS version 23, to compare the probiotic properties across isolates. Data were analyzed using descriptive and inferential statistical methods. The variations in biofilm formation among different Lactobacillus species were assessed using one-way analysis of variance (ANOVA). A two-stage least squares (2SLS) regression model was developed to explore the relationship between biofilm formation strength and microbial interactions, ensuring model reliability. The coefficient of determination (R^2) was calculated to evaluate the model's predictive power.[23, 24]

RESULTS

Table 1 presents the biofilm formation strength of various Lactobacillus species, categorizing them into no biofilm, weak, moderate, and strong formation. Table 2 and 3 shows the effect of temperature and pH on biofilm formation. Table 4 outlines the model description used for statistical analysis, identifying biofilm categories as predictors and instrumental variables. The results from Table 5's indicates model summary. Table 6 provides detailed coefficients of the variables in the model. Table 7's descriptive statistics summarize the central tendencies and variability of biofilm formation across isolates. The correlation matrix in Table 8 and 9 highlights the inverse relationship between strong biofilm formation and other categories. Finally, Table 10 offers the distribution parameters, showing how biofilm data fits a normal distribution. Figures 1 and 2 visually support these findings, with Figure 1 displaying a histogram of biofilm formation percentages and Figure 2 showing P-plots for estimated distribution parameters.

DISCUSSION

The distribution of biofilm formation strength among Lactobacillus species isolated from fermented cassava and corn samples highlights the variability in biofilm-forming abilities (Table 1). Lactobacillus fermentum (n=13) showed 46.1% strong biofilm formation and 7.7% no biofilm formation, while Lactobacillus plantarum (n=14) exhibited 42.9% strong and 7.1% no biofilm formation. These findings underscore

Table 1: Biofilm Formation in *Lactobacillus species* Isolated from Fermented Cassava and Corn Samples

Isolates	Biofilm formation strength			
	No biofilm n (%)	Weak n (%)	Moderate n (%)	Strong n (%)
Lactobacillus fermentum (n=13)	1(7.7%)	2(15.4%)	4(30.8%)	6(46.1%)
Lactobacillus ghanensis (n=9)	2(22.2%)	3(33.3%)	2(22.2%)	2(22.2%)
Lactobacillus delbrueckii (n=10)	2(20.0%)	2(20.0%)	4(40.0%)	2(20.0%)
Lactobacillus plantarum (n=14)	1(7.1%)	3(21.4%)	4(28.6%)	6(42.9%)
Lactococcus lactis (n=9)	2(22.2%)	4(44.4%)	2(22.2%)	1(11.1%)
Lactobacillus reuteri (n=8)	2(25.0%)	2(25.0%)	3(37.5%)	1(12.5%)
Lysinibacillus sphaericus (n=7)	3(42.9%)	2(28.6%)	2(28.6%)	0(00.0%)
Bacillus cereus (n=9)	2(22.2%)	2(22.2%)	4(44.4%)	1(11.1%)
Bacillus pacificus (n=11)	2(18.2%)	4(36.4%)	3(27.3%)	2(18.2%)

the association between strong biofilm formation and enhanced probiotic potential, contributing to microbial stability in the gastrointestinal tract. In contrast, *Lactobacillus ghanensis* and *Lactococcus lactis* demonstrated lower biofilm formation, likely influenced by genetic and environmental factors, such as substrate availability and pH, consistent with findings by Song et al.[28] Biofilm formation in *Lactobacillus* species is significantly influenced by temperature and pH. Table 2 shows that *Lactobacillus fermentum* and *L. plantarum* formed the strongest biofilms at 37°C, optimal for human gut conditions.[2] Biofilm production declined at 45°C, indicating stress. Table 3 reveals that pH 5.5–6.5 supported maximum biofilm formation, aligning with gut pH. Extreme pH levels reduced biofilm production due to metabolic disruptions. Statistical analysis (Table 4) revealed a highly significant predictive model ($R^2 = 0.987$, $p < 0.001$), aligning with Bajpai et al.[29], who used similar regression models to link microbial characteristics to biofilm variability. The significant F value (122.618, $p = 0.000$) underscores the robustness of these models. Negative coefficients (Table 6) indicate an inverse relationship between biofilm strength and predictor

Table 2: Effect of Temperature on Biofilm Formation

Isolates	Temperature (°C)			
	25	30	37	45
Lactobacillus fermentum, (%)	20.5	32.3	46.1	33
Lactobacillus plantarum, (%)	18	29.5	42.9	28
Lactococcus lactis, (%)	12.5	24.5	30	18.5
Lactobacillus ghanensis, (%)	14	22.2	27	20
Lactobacillus delbrueckii, (%)	13.5	19.5	25	18.5
Lactobacillus reuteri, (%)	15.6	21	32.5	22.4
Lysinibacillus sphaericus, (%)	16	23.2	33.8	23.5
Bacillus cereus, (%)	16.8	25.4	38.5	26.7
Bacillus pacificus, (%)	14.9	22.8	30.2	21.8

Table 3: Effect of pH on Biofilm Formation

Isolates	pH Level			
	4.5	5.5	6.5	7.5
Lactobacillus fermentum, (%)	28.5	39	46.1	31.2
Lactobacillus plantarum, (%)	26.2	35.1	42.9	28
Lactococcus lactis, (%)	18	25	30.5	22.5
Lactobacillus ghanensis, (%)	21.5	30	34.3	25.5
Lactobacillus delbrueckii, (%)	19	24.5	31	22.7
Lactobacillus reuteri, (%)	19.8	22.9	33.5	24.5
Lysinibacillus sphaericus, (%)	23	30.7	34.6	25.6
Bacillus cereus, (%)	24.5	31	35.5	26
Bacillus pacificus, (%)	21.4	28.5	32.4	27.4

Table 4: Two-stage Least Squares Analysis (Model Description)

Model Description		Type of Variable
Equation 1	**Strong**	Dependent
	Moderate	predictor & instrumental
	Weak	predictor & instrumental
	No biofilm	predictor & instrumental

Table 5: Model Summary

Equation 1	**Multiple R**	0.993
	R Square	0.987
	Adjusted R Square	0.979
	Std. Error of the Estimate	2.213

Table 6: ANOVA

		Sum of Squares	Df	Mean Square	F	Sig.
Equation 1	**Regression**	1802.009	3	600.670	122.618	0.000
	Residual	24.494	5	4.899		
	Total	1826.502	8			

Table 7: Coefficients

		Unstandardized Coefficients		Beta	T	Sig.
		B	Std. Error			
Equation 1	**(Constant)**	93.292	6.927		13.469	0.000
	Strong	-0.988	0.152	-0.387	-5.823	0.001
	Moderate	-0.927	0.144	-0.476	-6.455	0.001
	Weak	-0.949	0.130	-0.576	-7.311	0.001
	No biofilm	-0.888	0.076	-0.694	-11.639	0.000

Table 8: Descriptive Statistics

		No biofilm (%)	Weak (%)	Moderate (%)	Strong (%)	Isolates
Mean		20.0523	27.411	31.289	20.456	9
Std. Error of Mean		3.93630	3.0592	2.5879	5.0367	
Median		21.1000a	25.000a	29.333a	18.200a	
Std. Deviation		11.80890	9.1775	7.7636	15.1100	
Variance		139.450	84.226	60.274	228.313	
Skewness		.216	.691	.532	.814	
Std. Error of Skewness		.717	.717	.717	.717	
Kurtosis		1.708	-.156	-.826	-.046	
Std. Error of Kurtosis		1.400	1.400	1.400	1.400	
Range		42.83	29.0	22.2	46.1	
Minimum		.07	15.4	22.2	.0	
Maximum		42.90	44.4	44.4	46.1	
Sum		180.47	246.7	281.6	184.1	
Percentiles	**25**	15.5750b	21.050b	26.450b	11.333b	
	50	21.1000	25.000	29.333	18.200	
	75	23.9500	34.075	38.125	27.375	

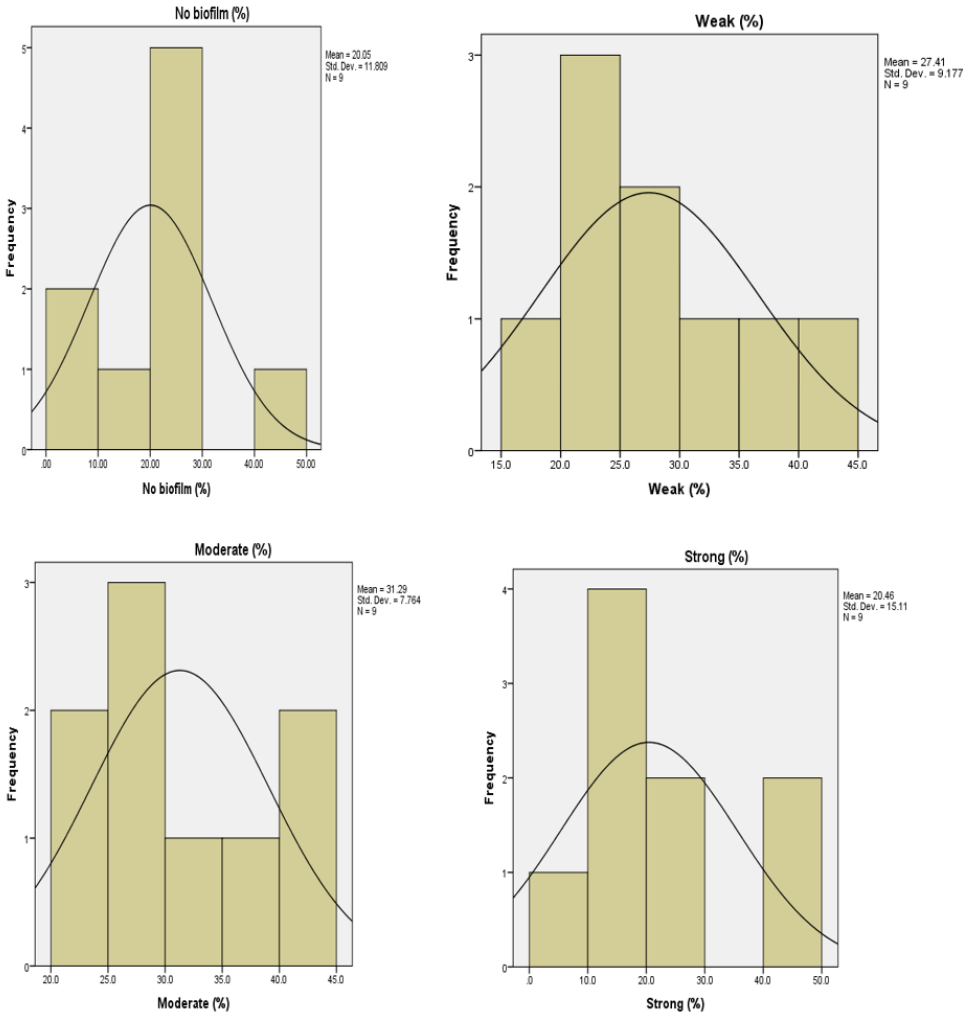

Figure 1: Histogram Showing the Percentage of Biofilm Formation in *Lactobacillus* Sp.

Table 9: Correlations

				No biofilm (%)	Weak (%)	Moderate (%)	Strong (%)
No biofilm (%)	Pearson Correlation			1	0.352	0.034	-0.914**
	Sig. (2-tailed)				0.353	0.930	0.001
	Sum of Squares and Cross-products			1115.600	305.389	25.114	-1304.199
	Covariance			139.450	38.174	3.139	-163.025
		95% Confidence Interval	Lower	1	0.352	0.034	-0.914
			Upper	1	0.352	0.034	-0.914
Weak (%)	Pearson Correlation			0.352	1	-0.654	-0.509
	Sig. (2-tailed)			0.353		0.056	0.161
	Sum of Squares and Cross-products			305.389	673.809	-372.809	-564.886
	Covariance			38.174	84.226	-46.601	-70.611
		95% Confidence Interval	Lower	0.352	1	-0.654	-0.509
			Upper	0.352	1	-0.654	-0.509
Moderate (%)	Pearson Correlation			0.034	-0.654	1	-0.123
	Sig. (2-tailed)			0.930	0.056		0.752
	Sum of Squares and Cross-products			25.114	-372.809	482.189	-115.754
	Covariance			3.139	-46.601	60.274	-14.469
		95% Confidence Interval	Lower	0.034	-0.654	1	-0.123
			Upper	0.034	-0.654	1	-0.123
Strong (%)	Pearson Correlation			-0.914**	-0.509	-0.123	1
	Sig. (2-tailed)			0.001	0.161	-0.752	
	Sum of Squares and Cross-products			-1304.199	-564.886	-115.754	1826.502
	Covariance			-163.025	-70.611	-14.469	228.313
		95% Confidence Interval	Lower	-0.914	-0.509	-0.123	1
			Upper	-0.914	-0.509	-0.123	1
**. Correlation is significant at the 0.01 level (2-tailed).							

Table 10: Estimated Distribution Parameters

Parameters		**No biofilm (%)**	**Weak (%)**	**Moderate (%)**	**Strong (%)**
Normal Distribution	**Location**	20.0523	27.4111	31.2889	20.4556
	Scale	11.80890	9.17748	7.76361	15.11002
The cases are unweighted.					

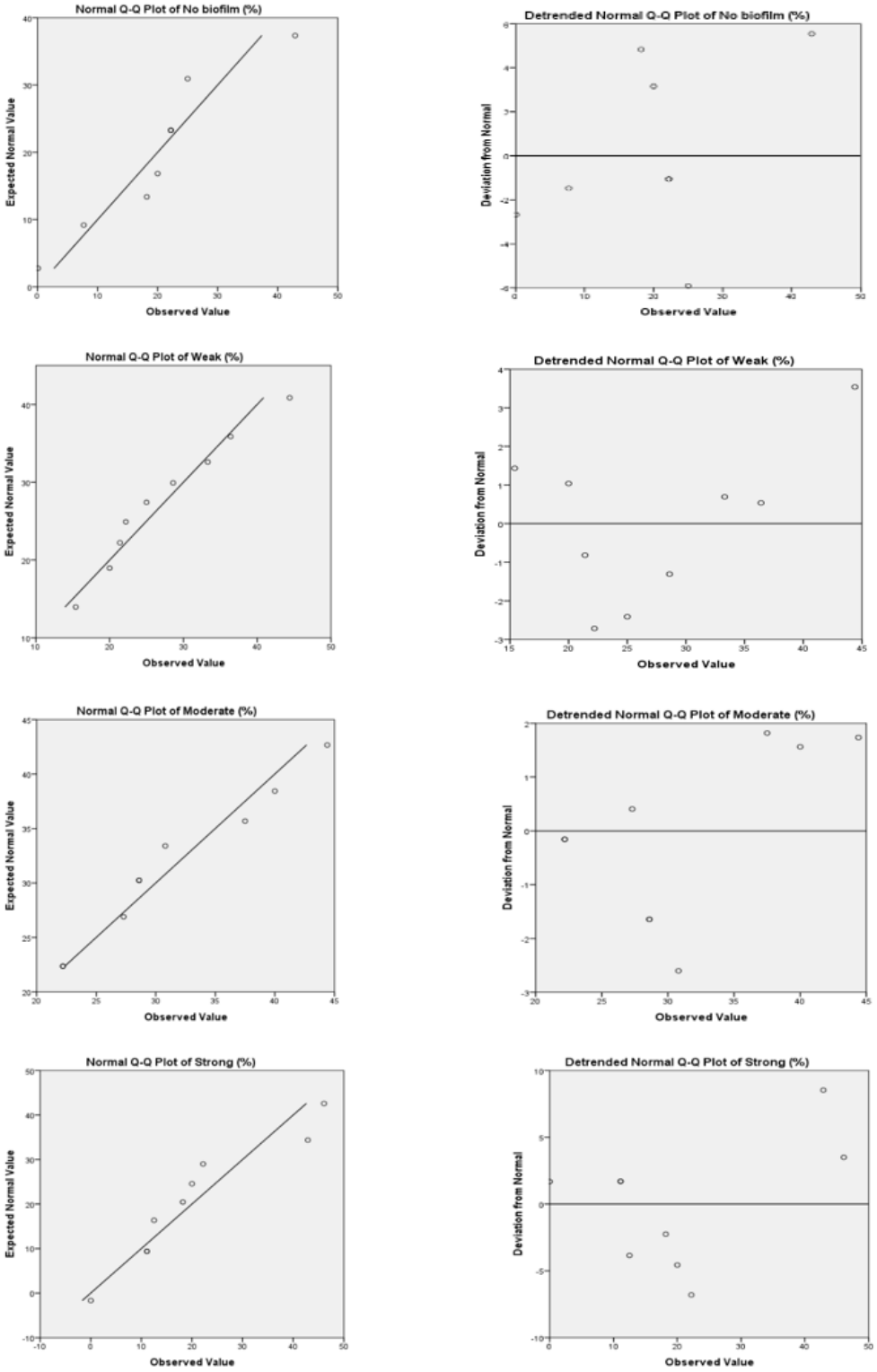

Figure 2: P-Plots for Estimated Distribution Parameters

variables, echoing Ahmed et al.[30], who noted metabolic and environmental stressors reduce biofilm formation.

Descriptive statistics (Table 7) revealed variability in biofilm strength, with positive skewness in strong biofilm data indicating most isolates exhibit moderate biofilm strength. Similar trends were reported by Fernández et al.[31] and Huang et al.[32] Distribution models (Figures 2 and 3) validate data robustness, supporting conclusions by Patel et al.[33] and Li et al.[34] The strong correlation ($r = -0.914$, $p = 0.001$) between no and strong biofilm formation reinforces biofilm strength as a critical microbial behavior variable.

CONCLUSION

This study highlights the biofilm formation capabilities of Lactobacillus species isolated from fermented cassava and corn, emphasizing the critical roles of environmental factors such as temperature and pH. Optimal biofilm production was observed at 37°C and pH 5.5–6.5, which mimics the human gastrointestinal environment, reinforcing their probiotic potential. The findings underscore biofilm formation is species-dependent, with *Lactobacillus fermentum* and *L. plantarum* demonstrating the strongest biofilm-forming abilities. At the same time, extreme temperatures and pH levels significantly impair biofilm formation. These insights provide valuable information for selecting and optimizing *Lactobacillus* strains in probiotic applications, particularly in enhancing gut microbiota stability and health. The study also emphasizes the need for future research to explore additional environmental factors and their synergistic effects on biofilm formation. Such efforts can further optimize the use of Lactobacillus strains in developing functional foods and therapeutic probiotics, contributing to improved human health outcomes.

Conflict of Interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical Statement

The study is proper with ethical standards, it was approved by the Department of Biological Sciences (Microbiology), Benson Idahosa University on 26th February, 2024.

Authors' Contribution

The research article was entirely written by OBA and ESA. Both authors contributed to the extensive literature search, analysis, and synthesis of findings across relevant studies. They collaborated on structuring the article and interpreting the research insights, aiming to provide a comprehensive overview of the topic

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