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Inhibition of biofilm formation in clinical isolates of *Staphylococcus aureus* by Lactobacillus spp. from soured milk

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ABSTRACT

Objectives: Staphylococcus aureus as a biofilm-forming pathogen has the ability to resist therapeutic treatments evident in prolonged disease burden. Thus, the aim of this study is to determine the anti-biofilm potential of lactic acid bacteria on biofilm forming *Staphylococcus aureus* from clinical samples.

*Methodology and Results***:** Seventeen (17) swab samples were collected from hospitalized patients and screened for the presence of biofilm forming *Staphylococcus aureus.* The percentages of *Staphylococcus aureus* obtained are 11.76%, 35.29% and 52.94% from body fluids, infected wounds and urinary crevices/implants respectively. The biofilm formation assay conducted showed that all the *Staphylococcus aureus* isolates were biofilm formers but with varying degrees of adherence, that is, weak and moderately adherent biofilm formers. The anti-biofilm effect of probiotic Lactic Acid Bacteria (LAB) isolated from soured milk on the isolates had highest percentage of moderate biofilm (60%) in urinary crevices isolates and weak biofilm formation (50%) from both body fluid and urinary crevices isolates. Comparative analysis of *Staphylococcus aureus* biofilm inhibition between probiotic from soured milk and pathogenic LAB showed that while the probiotic LAB significantly inhibited biofilm formation for all the *Staphylococcus aureus* isolates; S1-S17 ($p \leq 0.05$), the pathogenic LAB significantly inhibited biofilm formation in only four isolates; S10, S14, S16 and S17 ($p \leq 0.05$).

*Conclusions and application of findings***:** This study demonstrates the antibiofilm ability of probiotic LAB as better inhibitors than the pathogenic LAB species against a major virulence factor (biofilm formation) in *Staphylococcus aureus.* The antibiofilm potentials of LAB isolates can serve as alternatives to chemically synthesized agents in reducing or inhibiting pathogenic biofilm formations in treatment and management of infections in clinical settings**.**

Keywords: Biofilm; Lactic acid bacteria; Pathogenic; Probiotics; *Staphylococcus aureus*

INTRODUCTION

Biofilms are microbial communities made up of complex polysaccharides, proteins, lipids and extracellular DNA; they are protected by a layer of extracellular polymeric substance (EPS) produced within the microbial community. (Flemming and Wingender, 2010). Biofilm forming microbes are protected from host immunity, antimicrobial agents and other physiochemical properties in the environment, which aids expression of virulence factors by quorum sensing. (Flemming and Wingender. 2010; Rumbaugh *et al*., 2009; Karatan and Watnick, 2009). Naturally, the multiple species' communities in biofilm share their genetic material and fill up distinct niches within consortium (Watnick and Kolter 2000; Flemming *et al*., 2007). Bacterial communities established within the biofilm population matrix prevent other potential competitors from invading the same location thus ensuring long-term persistence (Hibbing *et al*, 2010). Biofilm-associated infections are of great concern in the medical community. Most bacterial infections associated with biofilm-forming organisms are usually device-related infections and malfunction of medical devices (Sanchez *et al*., 2017; Invernici *et al* 2018; Barzegari, *et al.*, 2020). Biofilms are also responsible for the increased antibiotic resistance because of the physical barrier to drug penetration, enzymatic inactivation of such drugs when consumed and lowered metabolic activity within the cells (Chao. and Zhang (2012; Høiby. 2011). Therefore, there is need for continuous investigations for therapies that specifically target the dispersal of biofilm matrix to planktonic mode of life (Fleming and Rumbaugh, 2017; Boles and Horswill 2008). Staphylococcus species are abundant in different environments with an inherent ability to form biofilms on both biotic and abiotic surfaces (Begun *et al.,* 2007; McCann *et a*l., 2008). Biofilm forming *Staphylococcus aureus* have the ability to attach to implants and other medical devices through direct interaction with the device's polymer surfaces or by establishing interaction with the human matrix, proteins leading to prolonged hospitalization and increase in cost of infection management (McConoughey *et al*., 2014; Ribeiro *et al*.,2012; Parvizi et al., 2010; Song *et al*., 2010). Lactic Acid Bacteria (LAB) which serve as probiotics are microorganisms, which confer health benefits to the host provided they are administered in the right dosage. These organisms interact with the host's gut microbiota and are useful in fighting off pathogenic biofilms (Barzegari *et al.,* 2020; Iannitti and Palmieri 2010; Wyszyńska, *et al*., 2015). Research findings and other high-throughput approaches have shown that probiotics present many health benefits to the host as they can modify the host mucosal and systemic immune responses, protecting the host against pathogens (Barzegari *et al.,* 2020; Zmantar *et al.,* 2008). Lactobacillus (lactic Acid Bacteria, LAB) as probiotics can modulate the microbiota and ecology of biofilms through growth inhibition, adhesion denudation and co-aggregation (Sanchez *et al.,* 2017; Barzegari *et al.,* 2020). Furthermore, it exerts antimicrobial activities against pathogens in the gastrointestinal (GI) tract by declining luminal pH, competing for nutrients and adhesion sites, as well as producing antimicrobial agents such as bacteriocins, hydrogen peroxide and organic acids. Thus, with the advances in the medical benefits of probiotics, this study seeks to determine the anti-biofilm potential of lactic acid bacteria from soured milk on clinical isolates of biofilm forming *Staphylococcus aureus* collected from hospitals within Enugu metropolis in South Eastern region of Nigeria.

MATERIALS AND METHODS

Sample collection: Sterile swab sticks were used to collect samples for the isolation of *Staphylococcus aureus* from patients with different hospitals. Samples were drawn from infected wounds, urinary crevices / implants and body fluids of the patients with their consent. Samples of soured milk, cheese and dried meat were collected from some selected areas in Enugu for the isolation of Lactic Acid Bacteria (LAB) using the methods by Reuben *et al*. (2019). The samples were properly labelled and transported within 1 (one) hour of collection using airtight sterile vial to the Microbiology Laboratory for immediate analysis. Tryptic Soy Agar (TSA) plates and Man Rogosa and Sharpe (MRS) broth and agar plates were prepared according to manufacturer's instruction.

Isolation of lactic acid bacteria: According to methods by Socransky, and Haffajee (2000), 10g quantity of each sample (soured milk, cheese and dried meat) were homogenized in 90 mL of Man Rogosa and Sharpe (MRS) broth and incubated at 37°C for 48 h under anaerobic conditions. Colonies were selected randomly and purified by sub-culturing and other biochemical tests. LAB isolates were confirmed as catalase-negative, Gram-positive, and rod-shaped bacilli. The purified isolates were stored in sterile TSB.

Haemolytic activity: Fresh cultures of LAB were streaked on Columbia agar plates, containing 5% (w/v) sheep blood, and incubated for 48 h at 37°C [24]. Plates were examined for signs of β-haemolysis (clear zones around colonies), α-haemolysis (green-hued zones around colonies) or γ-haemolysis (no clear zones around colonies). The assay was done in duplicate.

Isolation, characterization clinical isolates: The samples from the wound infection (WI), urinary crevices and implants (UC) and body fluids (BF) of the patients were smeared on Nutrient Agar (TSA) plates and incubated at 37^{o}C for 24 hours. Distinct colonies with milk/yellowish discolorations were sub cultured until pure colonies can be obtained. The isolates were transferred into a Staph-chromo agar (Merck). The Plates were incubated at 37°C for 24 hours.

Phenotypic Characterization of Slime Producing *S. aureus:* Biofilm formation were confirmed by a modified Congo Red Agar method as described by Freeman *et al*., (1999) and modified by Zmantar *et al*., (2008) Briefly, TS agar plates containing 50 g/L sucrose and 0.8 g/L Congo red were prepared and streaked with the *Staphylococcus aureus* isolates and incubated aerobically for 24 to 48 h at 37 ^{0}C. Positive results were indicated by black colonies with dry crystalline appearance. Weak slime producers usually remain pink, though occasional darkening at the centre of colonies might be observed.

Biofilm formation assay: The qualitative assay for biofilm formation performed according to the method described by Stewart and Costerton (2001) and G¨otz (2002) with certain modifications, was carried out by suspending overnight cultures of each isolate grown on Nutrient agar in Tryptic Soy Broth (TSB-BIOMED) and standardised to 0.1 OD_{595} in Tryptic Soy Broth (TSB). Each culture was incubated at 37°C for 24 hours. The cells were harvested at 4000 RPM (revolution per minute) for 10 minutes, and washed with sterile, distilled water. The harvested cell were suspended in TSB and standardised to 0.05 OD_{595}. Glass tubes were filled with 800 µL of TSB supplemented with 1% glucose (TSBg), an 80 µl of the standardized cultures of the isolated *Staphylococcus aureus* were inoculated in triplicates into the wells. The control contained 800 µL of the TSB without any inoculum. The tubes were incubated at 37°C for 48 hrs. After incubation, the tubes were washed three times with sterile, distilled water. The bacterial cells that had adhered to the surface of the glass tubes were fixed by treating with 96% ethanol

for 5 minutes at room temperature. After removal of alcohol, the biofilms were stained with 800 µL 1.0% crystal violet (CV) for 15 minutes, the tubes were then washed three times with sterile distilled water, and the tubes were left to air dry for another 15 minutes. Finally, 800 µL of 33% glacial acetic acid was added to each well, and the OD was read using a spectrophotometer at 570 nm. The test was considered positive when there was an adherent layer of stained material on the inner surface of the tube. The adherence was estimated as absent (0), weak (+), moderate (++), or strong (+++). The presence of stained material at the liquid–air interface was not considered indicative of biofilm formation. The test was repeated three times for each strain.

Antibiofilm activity of LAB: The anti-biofilm ability of the LAB was determined based on the dispersion of preformed biofilm of *Staphylococcus aureus*. Biofilm of *Staphylococcus aureus* were developed in glass tube (13x100 mm) by adding 800 µl of autoclaved TS broth along with 80 µl of overnight grown *Staphylococcus aureus* culture having 0.05 OD_{595}. After 24 h incubation at 37^0C, non-adherent cells were removed by gentle pipetting without disrupting biofilm. An 80 µl of the isolated lactobacilli at 0.05 OD_{595} was added along with the 800 µl of autoclaved TS broth. In the control tubes, instead of the LAB+TSB, 880 µl of autoclaved TS broth was added. The plates were incubated at 37◦C for 48 h. The experiment was conducted in triplicates. Calculation of biofilm formed was done using the formula:

% biofilm inhibition=100-[OD @595 in the presence of *Staphylococcus aureus* and LAB*100] OD @ 595 in the presence of tryptic soy broth and LAB (Control)

Statistical analysis*:* Pearson's chi-squared test were employed at the $p < 0.05$ significance level to compare differences between groups. Statistical analysis was performed with SPSS Windows software, version 22.0 for the relationship between the dependent variables and independent variable.

RESULTS

Isolation of *Staphylococcus aureus:* Of the seventeen (17) *S. aureus* isolates, 35.29% was from infected wound samples, 11.76% from body fluids, and 52.94% were isolated from urinary crevices and implants. These isolates where biochemically identified. *S. aureus* isolates showing gamma haemolysis also produced hydrogen sulphide.

Table 1: Biochemical identification of the *S. aureus* isolates

No of Isolates	GR	MF	UU	CAT	COA	Dns	OXI	BH	GP	H_2S	PRE ORG
17	++	++	++	++	++	++	--	γ	--	--	*Staphylococcus aureus*

GR= Gram reaction, **MF=** Mannitol fermentation, **UU=** Urea utilization, **CAT=** Catalase, **COA=** Coagulase, **Dns=** Dnase, **OXI=** Oxidase, **BH=** Blood haemolysis, **GP=** Gas production, **H_2S=** H_2s production, **PRE ORG =** Presumptive organisms, **++ = positive,** -- = **negative** and γ = gamma haemolysis

Slime-Producing *S. aureus* Phenotypic Characterization: Out of the seventeen (17) isolates, four showed visible slime production and thick black coloration with dry crystalline appearance almost covering the modified Congo red agar; eight showed a moderate black coloration on the Congo red agar. While the remaining five maintained their pink to red coloration, which was darkened after an additional 48 hours. Positive results were indicated by black colonies with dry crystalline appearance.

Isolation of lactic acid bacteria: The LAB isolates were obtained from sour milk and showed varying degrees of haemolysis.

Fig 1: LAB isolates

Biofilm Formation Assay: The classification of biofilm formation and adherence was done by subtracting mean values of OD obtained for blank tests from the mean values of OD obtained for each test strain. The clinical isolates used in this work were classified into four categories according to Stepanovic *et al*. (2007); non-adherent (OD < ODc); weakly adherent (ODc < OD < 2xODc); moderately adherent (2xODc < OD < 4xODc); strongly adherent (4xODc < OD); with ODc: the cut-off OD (three standard deviations above the mean OD of the blank test). The averaged OD values and standard deviations were calculated using SPSS version 23.

Table 2: Classification of biofilm formation abilities by microtiter/glass tube method

Cut-off value calculation	Mean of OD values results	Biofilm formation ability	Percentage of biofilm (%)
OD > 4×ODc	OD>2.485	Strong (+++)	0
2×ODc < OD ≤4×ODc	1.242<OD≤2.485	Moderate(++)	88.24
ODc< OD ≤ 2×ODc	0.621< OD ≤ 1.242	Weak(+)	11.76
OD ≤ 0.621	OD ≤ 0.621	None(0)	0

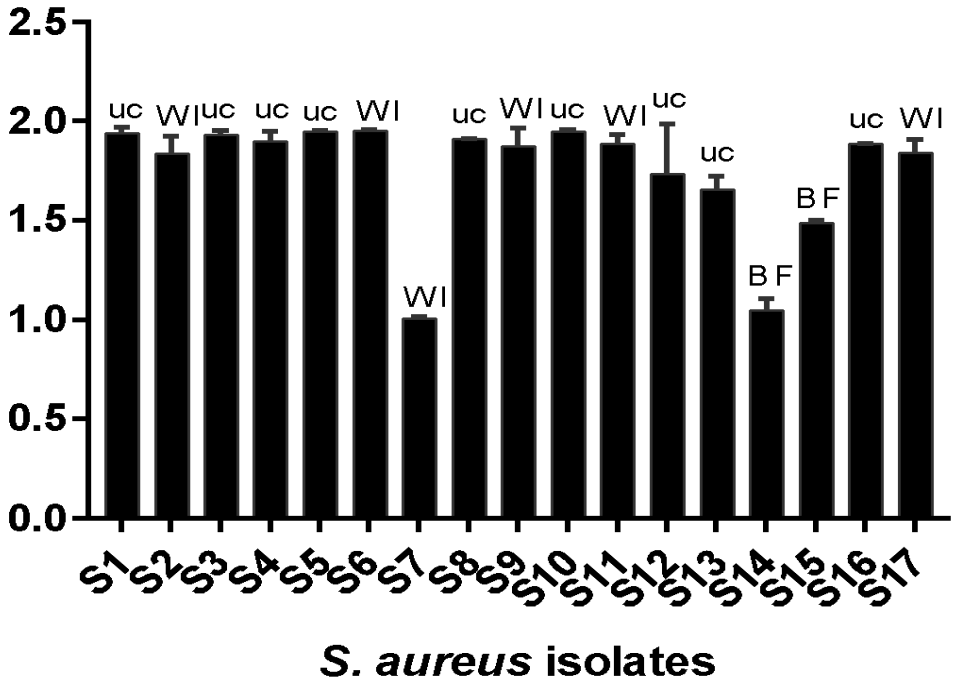

Fig 2: Optical Density of Biofilm Formed by *S. aureus* Isolates from Hospitalized Patients. (UC: urinary crevices and implants, WI: wound infection, BF: body fluids)

Antibiofilm effect of lab on the *Staphylococcus aureus* isolates: Out of the seventeen biofilm forming isolates, five (S13-17) were inhibited by a degree less than ODc (0.621) by the four LABs used. Two (S4 and S7) were inhibited greater than or equivalent to ODc, while the remaining ten isolates were inhibited to the degree less than twice the ODc.

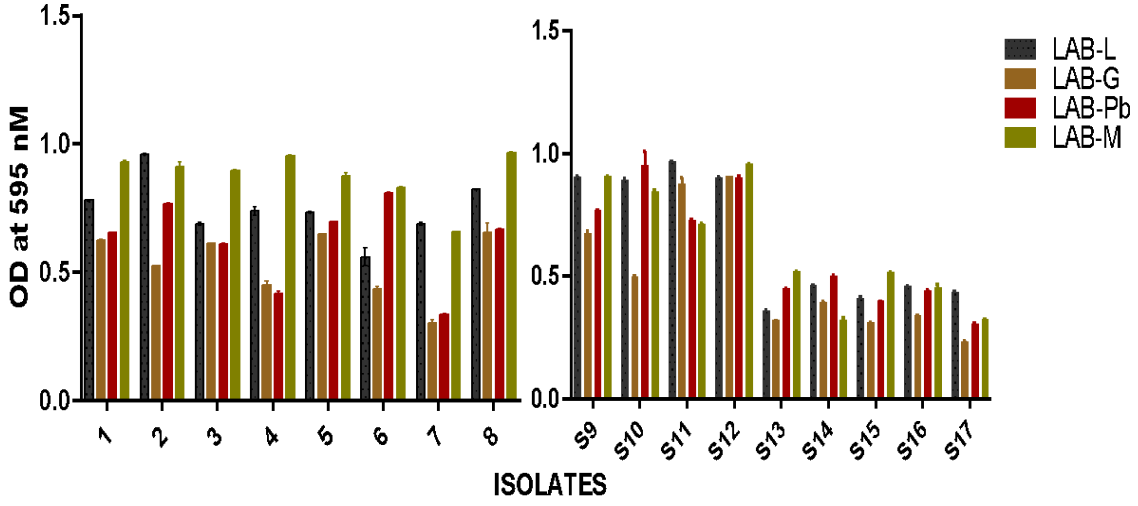

Figure 3: Optical Density of Biofilm Formed by *S. aureus* Isolates from Hospitalized Patients treated with different Lactobacillus Isolated from Soured Milk

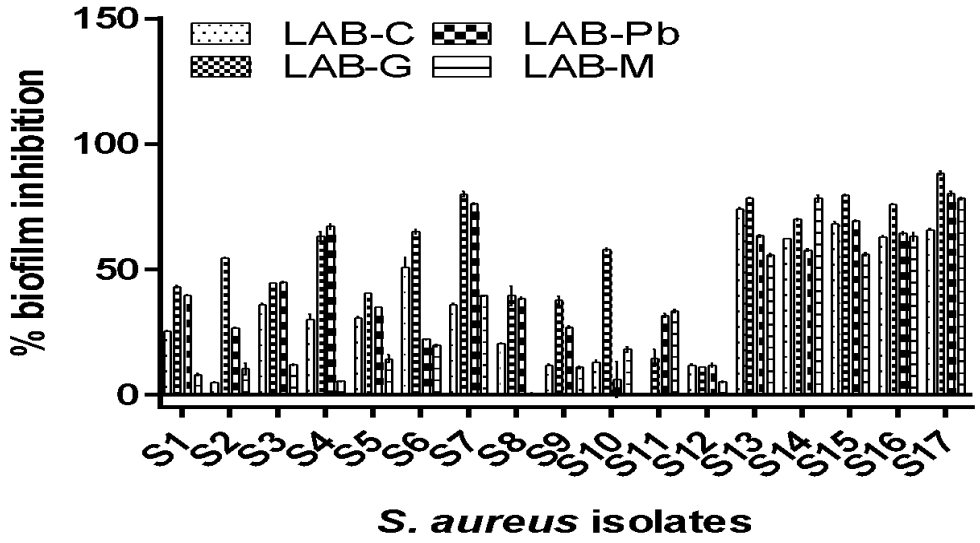

Figure 4: Percentage biofilm inhibition of Staphylococcus Isolates from Hospitalized Patients treated with different Lactobacillus Isolated from Soured Milk.

TABLE 3: biofilm inhibition using the optical density of the isolates after inhibition with different potential probiotic LAB isolates using Turkey test at $p<0.05$.

Inhibitors	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17
LAB-C	0.78± 0.00^{C}	0.96± 0.00^{D}	0.69± 0.01^{B}	0.74± 0.02^{B}	0.73± 0.00^{C}	0.56± 0.03^{B}	0.69± 0.01^{D}	0.82± 0.00^{B}	0.90± 0.01^{C}	0.89± 0.01^{B}	0.96± 0.01^{C}	0.90± 0.01^{A}	0.36± 0.01^{B}	0.46± 0.00^{C}	0.41± .01^{B}	0.46± 0.01^{B}	0.43± 0.01^{C}
LABG	0.63± 0.00^{A}	0.53± 0.00^{A}	0.61± 0.00^{A}	0.45± 0.02^{A}	0.65± 0.00^{A}	0.43± 0.01^{A}	0.30± 0.01^{A}	0.66± 0.03^{A}	0.67± 0.01^{A}	0.50± 0.00^{A}	0.87± 0.03^{B}	0.91± 0.00^{A}	0.32± 0.00^{A}	0.39± 0.00^{B}	0.31± 0.00^{A}	0.34± 0.00^{A}	0.23± 0.01^{A}
LABPB	0.66± 0.00^{B}	0.77± 0.00^{B}	0.61± 0.00^{A}	0.42± 0.01^{A}	0.70± 0.00^{B}	0.81± 0.00^{C}	0.34± 0.00^{B}	0.67± 0.00^{A}	0.77± 0.00^{B}	0.95± 0.06^{B}	0.73± 0.01^{A}	0.90± 0.01^{A}	0.45± 0.00^{C}	0.50± 0.00^{D}	0.40± 0.00^{B}	0.44± 0.01^{B}	0.30± 0.01^{B}
LABM	0.93± 0.00^{D}	0.91± 0.02^{C}	0.90± 0.00^{C}	0.95± 0.00^{C}	0.88± 0.01^{D}	0.83± 0.00^{C}	0.66± 0.00^{C}	0.97± 0.00^{C}	0.91± 0.00^{C}	0.84± 0.01^{B}	0.71± 0.01^{A}	0.96± 0.00^{B}	0.52± 0.01^{D}	0.32± 0.01^{A}	0.51± 0.00^{C}	0.45± 0.01^{B}	0.32± 0.00^{B}

*Values of OD of different isolates of staphylococcus after inhibition with free cell supernatant are represented as mean plus or minus (±) standard deviation. Means with different superscripts (letters) within the groups (in column) are statistically different at $p\leq0.05$ using turkey test.

The significance of different probiotics (Four LABs used in this study) whole-cell biofilm inhibition capacity against different *Staphylococcus aureus* biofilm was investigated using the percentage biofilm inhibition of the *Staphylococcus aureus* isolates inhibited, in a one way ANOVA; and there was no significance i.e. different cell-free of the probiotics inhibited the biofilms of the seventeen isolates of *Staphylococcus aureus* at the same or similar capacity with a known pathogenic *Lactobacillus spp* in all the seventeen isolates at p-value ≤ 0.05 except in S10, S16 and S17, (table 7)

DISCUSSION

Treatment of chronic infections caused by *S. aureus* impose substantial financial burden globally due to the organisms ability to form biofilm (Stewart and Costerton 2001; G¨otz, 2002; Kim *et al*., 2008). This study investigated the ability of *S. aureus* strains isolated from clinical samples to form biofilm. There was an evidence of slime production in the seventeen *S. aureus* isolates used but the degree of production varied. Four (4) 23.5% of the *S. aureus* isolates (S3, S5, and S13-14) showed highly visible slime production with a thick black coloration and dry crystalline appearance after 48 hours; 8 (47.1%) of the *S. aureus* isolates showed black coloration with a moderate appearance after 48 hours, while 5 (29.4%) maintained their pink to red coloration but darkened after an additional 48 hours. The isolates from urinary crevices and implants and those from wound infections produced black coloration on Congo red supplemented TS agar medium; this is in accordance with Agarwal and Jain (2013) who opined that isolates showing biofilm producing potential occurred more among invasive (from blood) and colonizing (from intravenous device). In the study, Agarwal and Jain grouped *S. aureus* in three categories where they revealed that isolates showing biofilm producing potential are more in invasive and colonizing isolates than in group of commensal isolates (from skin or nose). The percentage ratio of biofilm formation in the isolates was in the following order: urinary crevices (60.00%), infections (33.33%) and body fluids (6.67%), whereas 50.00% of weak biofilm was from body fluid and 50.00% was from urinary crevices. Bridier *et al*. (2010) reported that *S. aureus* strains from different sources produced biofilms with high bio-volumes. Piechota *et al*., (2018) also reported that production of strong biofilm by *S. aureus* is mostly associated with isolates from contaminated medical implants, respiratory tract and/or medical devices because of environmental selection. These studies, corroborates the moderate biofilm in a glass tube method observed in this study. There was significant difference statistically between the *S. aureus* biofilm inhibition by the four potential probiotics except in *S. aureus* S14 (P=0.002) where there was a difference statistically in the inhibition by the potential probiotics of LAB isolated in this study and the pathogenic LAB got from a depository of the department of Microbiology University of Nigeria Nsukka, the inhibition of *S. aureus* biofilm by the four potential probiotics LAB isolates showed no difference statistically when compared with other pathogenic LABs using the optical density of the residual biofilm. Interestingly, there was significant variation also for the percentage of biofilm inhibited in the seventeen *S. aureus* isolates when the different potential probiotics of LAB isolated in this study where placed side-by-side. However, when compared with other pathogenic LABs, there was significant variation only in *S. aureus* isolates S10 (P=0.027), S16 (P=0.005) and S17 (P=0.002). Most inhibition occurred in the *S. aureus* biofilms from urinary crevices and body fluids. Biofilm formation depends on many factors such as environment, availability of nutrients, and above all the presence of the biofilm-associated genes and their expression (Neopane *et al.*, 2018; Kot, *et al.*, 2018).

CONCLUSION AND APPLICATION OF RESULTS

The findings of this study confirm the anti-biofilm properties of LAB against *Staphylococcus aureus* isolates from clinical samples. Thus, making the use of probiotics as a treatment option in the management of Staphylococci infections, treatment of diarrhoea, boosting the immune system by inhibiting the growth and adhesion of a range of enteropathogen. Further studies using the cell free supernatant of the probiotics will help to identify the compound(s) responsible for the anti-biofilm activity and possible synergistic combinatorial outcomes with other medicinal plants.

CONFLICT OF INTEREST

The authors of this paper declare that there is no conflict of interest.

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