Biofilm Inhibitory Effects of Lactobacillus Spp Against Streptomycin-resistant Uropathogenic Escherichia Coli

NWANEKWU KENNETH EMEKA
Department of Microbiology, Faculty of Biological Science, Imo State University, Owerri, NIGERIA

Abstract: The biofilm inhibitory effects of *Lactobacillus* spp against Streptomycin-resistant uropathogenic *Escherichia coli* (UPEC) were evaluated using the crystal violet test method. *Lactobacillus* spp were isolated from milk samples while fifty strains of Uropathogenic Escherichia coli were isolated from urine samples from Urinary Tract Infection patients attending Federal Medical Centre (FMC) Owerri, Nigeria. Ten of the *E. coli* strains resistant to streptomycin antibiotics were screened for their susceptibility to antibiofilm effect of Lactobacillus secondary metabolites extracts. From the result obtained, only one of the *E. coli* strains was susceptible while nine strains were resistant. This result shows clearly that the metabolite extracts from *Lactobacillus* sp were not effective in the antibiofilm activity of the *E. coli* strains and thus not a good candidate for the management of UTI caused by *E. coli*.

Keywords: Uropathogenic, Antibiofilm, UTI, Lactobacillus, *E. coli*

Received: July 2, 2022. Revised: September 14, 2023. Accepted: October 19, 2023. Published: November 27, 2023.

1. Introduction

Uropathogenic *Escherichia coli* (UPEC) is a nonsporulating, flagellated, facultative anaerobic Gram-negative rod belonging to the family *Enterobacteriaceae* (Yi-Te *et al*., 2020). It is the most significant causative agent of UTIs in humans accounting for about 75% of cases (Flores-Mireles *et al*., 2015). It is also a major food contaminant causing serious food spoilage and food borne infection (CDC 2012). *E. coli* has the capability to cause cause UTI and other diseases because of the various virulence factors it possesses. These factors include, acid tolerance, toxin production and biofilm formation (Wiles *et al*., 2008). Biofilm is a mass of microbial cells attached to a surface and enclosed in a matrix of polysaccharide (Donlan, 2002). Biofilms formation serves as a survival mechanism for bacteria during extreme environmental conditions such as nutrient deficiency and antimicrobial actions (Nandakumar *et al*., 2013). These organisms have also been found to harbor a large number of antibiotic inactivating enzymes such as beta-lactamases leading to antimicrobial resistance (Davies & Davies 2010). Several studies have reported cases of antimicrobial resistance among UPEC especially among commonly used antibiotics such as ciprofloxacin, trimethoprim-sulphamethoxazole, streptomycin among others (Ali *et al*., 2016; Neupane *et al*., 2016).

Streptomycin is the first discovered aminoglycoside antibiotic, originally isolated from the bacteria *Streptomyces griseus*. It has activity against several aerobic gram-negative bacteria including *E. coli* (Zhu *et al*., 2001). Its broad-spectrum activity against gram-negative and gram-positive bacteria has been greatly diminished, largely due to developing antibiotic resistance (Daniel, 2005). The mechanism of resistance appears to be associated with the inhibition of its active transport into the bacterial cell. Commonly resistant bacteria include *Enterobacteriaceae* and most *Streptococci* species (Akhtar *et al*., 2016; Azam *et al*., 2019). Streptomycin is a drug of choice in the treatment of *E. coli* infections. They have been used in several local communities in the management of UTI caused by *E. coli*. However, there have been several cases of treatment failures and incidence of streptomycin resistance by *E. coli* and other gram-negative organisms lately, necessitating the need for other natural products alternatives.

Lactobacilli are widespread in nature and reside in a variety of natural habitats, ranging from plants to the mammalian oral, gastrointestinal or vaginal cavities (Kenreigh &Wagner 2006). Lactobacilli are known for their ability to inhibit the growth of bacteria due to the production of antimicrobial materials such as bacteriocins, biosurfactants and lactic acid (Soleimani *et al*., 2010). These secondary metabolites produced by Lactobacillus have been

shown to possess antibacterial activity against most pathogenic organisms (Mejlholm and Dalgaard 2015). However, to combat the problem of antimicrobial resistance, these metabolites are used against the virulence factor (biofilm formation) which is responsible for the pathogenicity of the disease rather than the inhibitory activity against the organism. Thus, the present study is therefore aimed at evaluating the antibiofilm activity of metabolites secreted by Lactobacilli against Streptomycin-resistant uropathogenic *E. coli* (UPEC).

2. Materials and Methods

Escherichia coli

The Uropathogenic *E. coli* strains were isolated from patients with urinary tract infection in the clinical diagnostic Laboratory of Federal Medical Centre Owerri, Nigeria using standard bacteriological methods. Urine samples from 50 women were collected in sterile specimen screw-capped bottles and transported to the Lab immediately for analysis. One milliliter of the urine specimen was inoculated into 19mL molten agar and poured into a sterile petri dish. The agar was allowed to solidify and then incubated at 37^{o}C for 24hours. After incubation, the colonies formed were subjected to conventional biochemical tests to confirm the presence of *E. coli* strains.

Lactobacillus spp

Lactobacillus spp were isolated from different milk samples according to the method described by Mahsa *et al* (2017). One hundred (100) ml of the liquid milk samples were collected in sterile conical flasks and allowed to ferment at room temperature for 3 days. Ten-fold serial dilutions of the samples were made and 0.1ml of suitable dilution inoculated unto MRS agar. The pH of the medium was adjusted to 5.5 by adding HCl. The set plates were incubated anaerobically at 35^{o}C for 48hours. Colonies were tested for catalase activity. Catalase negative organisms were sub-cultured onto fresh sterile MRS Agar to obtain pure culture. The isolated microorganisms were sub-cultured unto a maintenance culture medium of MRS broth containing 12% v/v glycerol. This was incubated at 30^{o}C until growth was detected and then stored at 4^{o}C in refrigerators.

2.1 Preparation of Lactobacillus metabolite extract

Metabolites extracts from the *Lactobacillus* spp was prepared by the method described by Rao et al., (2015). Broth cultures of all the LAB isolates were first prepared by simply inoculating a loopful of culture into fresh 20ml MRS broth in 25ml sterile bottles and incubated at 35^{o}C in an anaerobic jar for 72 hours. The 72hour broth culture of *Lactobacillus* spp was used in the preparation of crude extract. Ten mil (10ml) of the LAB broth culture was transferred into tubes and centrifuged at 5000 revolution per minute (r.p.m) for 15 minutes to obtain clear sedimentation of the pellets. The supernatant was decanted into separate containers. The supernatant fluid was adjusted to pH 6.5 by adding NaOH and then treated with 5mg/ml catalase. The supernatant fluid was then filter sterilized through a 0.45µm pore size cellulose acetate filter. The product was designated as LAB metabolite extract.

2.2 Antibiotics susceptibility testing

The antibiotics susceptibility test was carried out using the Agar disk diffusion method (Syukur, *et al*., 2014). A volume of 100 µl of an overnight culture of each UPEC isolate on Mueller-Hinton broth with the turbidity of 0.5 McFarland was streaked on Mueller-Hinton agar plates. The routinely used 10 antibiotic discs, including Reflacin, Nalidixic acid, Augmentin, Gentamycin, Ampicillin, Ofloxacin, Streptomycin, Septrin, Ampicillin and Ciprofloxacin were placed on the surface of the inoculated plates. The plates were incubated at 37° C for 24 hr.

2.3 Investigation of the anti-adhesive effect of lactobacilli supernatant

To evaluate the anti-adhesive effect of the Lactobacilli, polystyrene microtiter plate 100 was used. First, 75 µl of the lactobacilli supernatant and then 75 µl culture suspension of UPEC were added to the wells. The microtiter plates were incubated at 37°C for 24 hours. Each of UPEC (without lactobacilli) was poured into the control wells. Then, the contents of the wells were removed and each well was washed three times by PBS. Ethanol 96% w/w^{-1} (for 15 min) and 2% w/w^{-1} crystal violet (for 10 min) were used for stabilizing the cells and staining, respectively. Then, the polystyrene microtiter plate was rinsed with a gentle stream of

water. When the wells were dried by exposing to the air, 33% w/w^{-1} acetic acid was added to the wells as a solvent, and optical absorbance was measured at 492 nm for each well using spectrophotometer. The test was carried out in duplicate (Mahsa *et al*., 2017).

3. Results and Discussion

3.1 Isolation and Identification of *Lactobacillus* spp

Morphological and physiological characteristics of the Lactobacillus isolates were carried out and presented in Table 1. The isolates were motile, Gram-positive, non-spore forming and catalase-negative rods. These are typical characteristics of Lactobacilli as commonly isolated from milk and other fermented products (Crowley *et al*., 2013).

3.2 Isolation and Identification of *E. coli* strains

Echerichia coli strains were isolated and identified by their morphological and physiological characteristics and presented in Table 2. All the isolates were Indole and MR positive, VP, Citrate and Urea negative respectively. These characteristics represent typical physiological properties of *E. coli* as reported in previous research (Bukh *et al*., 2009; Tenaillon *et al*., 2010).

Table 1: Morphological and Biochemical Characteristics of Lactobacillus Isolates

Isolate	Gram	Shape	Spore formation	Motility	Catalase	Nitrate	Growth at 5% NaCl	Suspected Organism
I	+	Rod	-	+	-	-	+	*Lactobacillus acidophilus*
II	+	Rod	-	+	-	-	+	*Lactobacillus acidophilus*
III	+	Rod	-	+	-	-	+	*Lactobacillus acidophilus*

Table 2: Morphological and Biochemical Characterization of *E. coli* strains

Isolate	Gram	Shape	Motility	MR	VP	Indole	Citrate	Urea	Suspected Organism
I	-	Rod	+	+	-	+	-	-	*Escherichia coli*
II	-	Rod	+	+	-	+	-	-	*Escherichia coli*
III	-	Rod	+	+	-	+	-	-	*Escherichia coli*
IV	-	Rod	+	+	-	+	-	-	*Escherichia coli*
V	-	Rod	+	+	-	+	-	-	*Escherichia coli*
VI	-	Rod	+	+	-	+	-	-	*Escherichia coli*
VII	-	Rod	+	+	-	+	-	-	*Escherichia coli*
VIII	-	Rod	+	+	-	+	-	-	*Escherichia coli*
IX	-	Rod	+	+	-	+	-	-	*Escherichia coli*
X	-	Rod	+	+	-	+	-	-	*Escherichia coli*

3.3 Antibiotics susceptibility test

Nine antibiotics were tested against the *E. coli* strains isolated and the results presented in Table 3. From the results obtained, the organisms showed varying reactions to the tested antibiotics. The antibiotics Reflacin, Ciprofloxacin, Augmentin, Gentamycin and Ampicillin inhibited the growth of the organisms, while the *E. coli* strains were resistant to Oflocxacin, Septrin, Nalidixic acid and Streptomycin. This result is in line with previous reports of the established resistance of *E. coli* to a number of antibiotics including those reported in this

study (Osungunna & Onawunmi 2018). Ten of the *E. coli* strains tested were all remarkably resistant to Streptomycin while been susceptible to Ciprofloxacin. This is in clear disagreement with the report of Mahsa *et al*., (2017) which reported that the biofilm forming strains of *E. coli* were resistant to Ciprofloxacin. This could be as a result of the degree of exposure and use of the various antibiotics in the treatment of UTI in different countries and geographical locations. It however agrees with Dabo *et al.*, (2019) and Rafique *et al*., (2020) who reported *E. coli* resistance to Streptomycin.

3.4 Antibiofilm-adhesive effect of lactobacilli supernatant against *E. coli* isolates

Results of the antibiofilm adhesive effect of Lactobacillus supernatant against *E. coli* strains are presented in Figure 1. The figure showed that the Lactobacilli metabolites did not inhibit or affect the biofilm adhesive capabilities of nine of the *E. coli* strains, while only one *E. coli* strain EC1among the ten strains were susceptible to the effects of the Lactobacilli. The EC5 strain was the most resistant isolate to Lactobacilli metabolite. This shows that the Lactobacilli metabolites are not effective in inhibiting the effect of Uropathogenic *E. coli* biofilm. The results obtained in this study did not agree with the report of Mahsa *et al*., (2017) and Abedi *et al.,* (2013) which reported that probiotic Lactibcilli had anti-adhesive effect.

Table 3: Antibiotic susceptibility test

Antibiotic	EC1	EC2	EC3	EC4	EC5	EC6	EC7	EC8	EC9	EC10
Reflacin	+	+	+	+	+	+	+	+	+	+
Ciprofloxacin	+	+	+	+	+	+	+	+	+	+
Augmentin	-	+	+	+	+	+	+	+	+	+
Gentamycin	+	+	+	+	+	+	+	+	+	+
Ampicillin	-	+	+	+	+	+	+	+	+	+
Ofloxacin	+	-	-	-	-	-	-	-	-	+
Streptomycin	-	-	-	-	-	-	-	-	-	-
Septrin	-	-	-	+	-	-	-	-	+	-
Nalidixic acid	-	+	-	-	-	-	+	-	-	-

+ Susceptible; - Resistant

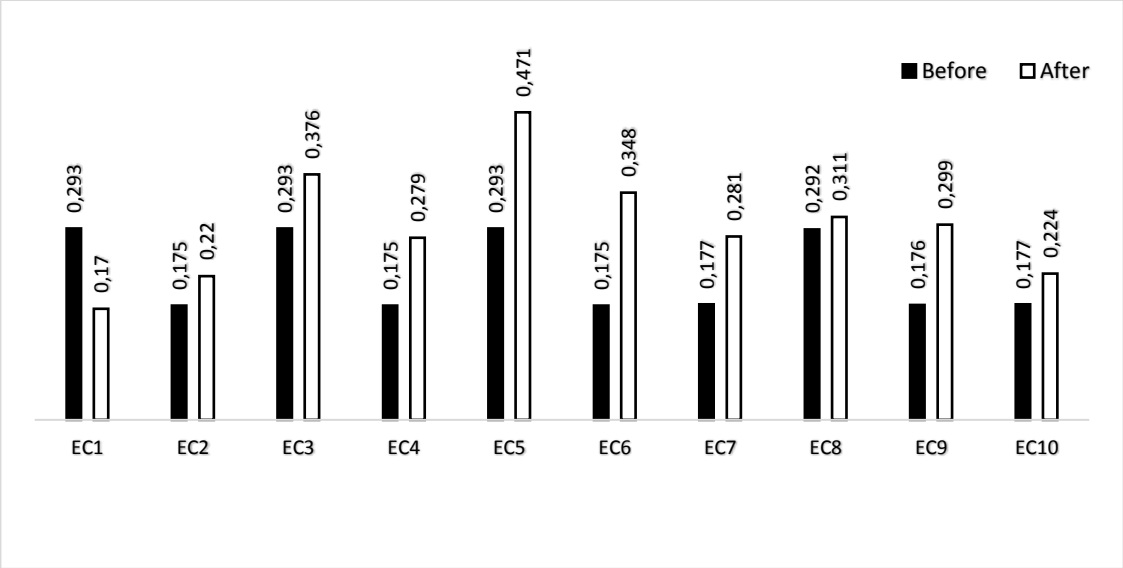

Fig 1: Antibiofilm adhesive effect of Lactobacilli supernatant against *E. coli* isolates

4. Conclusion

Based on the findings of this study, it is therefore imperative to say that secondary metabolite extracts from *Lactobacillus* spp doesn't have antibiofilm effects against Streptomycin-resistant Uropathogenic *E. coli* and as such cannot be used in the management of UTI as alternative natural product to antibiotics.

There is therefore the need to continue the search and screening of other natural product candidates such as medicinal plants and other probiotic organisms as potential and alternative sources of antibiotics in the management and treatment of microbial infections and combat the incidence of antibiotics resistance. Further work will be done in looking at medicinal plant extracts against biofilm formation and other virulence factors produced by antibiotic resistant *E. coli*.

References

[1]. Abedi, D., Feizizadeh, S., Akbari, V. & Jafarian-Dehkordi, A. (2013). In vitro antibacterial and anti-adherence effects of Lactobacillus delbrueckii subsp bulgaricus on Escherichia coli. Res Pharm Sci., 8(4): 260-268

[2]. Akhtar, F., Rabbani, M., Muhammad, K., Younus, M., Ghafoor, A., Sheikh, A. A., et al. (2016). Comparative antibiotic resistance profile of the multidrug resistant E. coli isolated from commercial and backyard poultry. J. Anim. Plant Sci. 26, 1628–1632.

[3]. Ali, I., Rafaque, Z., Ahmed, S., Malik, S. & Dasti, J.I. (2016). Prevalence of multi-drug resistant uropathogenic Escherichia coli in Potohar region of Pakistan. Asian Pac J Trop Biomed., 6(1):60–6.

[4]. Azam, M., Mohsin, M., Sajjad-Ur-Rahman, and Saleemi, M. K. (2019). Virulence-associated genes and antimicrobial resistance among avian pathogenic Escherichia coli from colibacillosis affected broilers in Pakistan. Trop. Anim. Health Prod. 51, 1259–1265. doi: 10.1007/s11250-019-01823-3

[5]. Bukh, A.S., Schonheyder, H.C., Emmersen, J.M., Sogaard, M., Bastholm, S. & Roslev, P. (2009). Escherichia coli phylogenetic groups are associated with site of infection and level of antibiotic resistance in community-acquired bacteraemia: A 10 year population-based study in Denmark. J Antimicrob Chemother., 64(1): 163-168. doi: 10.1093/jac/dkp156.

[6]. CDC (2012). Escherichia coli. National Center for Emerging and Zoonotic Infectious Diseases. Retrieved 2 October 2012

[7]. Crowley, S., Mahony, J., & van Sinderen, D. (2013). Current perspectives on antifungal lactic acid bacteria as natural bio-preservatives. Trends in Food Science & Technology, 33(2), 93-109. http://dx.doi. org/10.1016/j.tifs.2013.07.004.

[8]. Daniel, T.M. (2005). Selman Abraham Waksman and the discovery of streptomycin. Int J Tuberc Lung Dis., 9(2):120-2.

[9]. Davies, J. & Davies, D. (2010). Origins and evolution of antibiotic resistance. Microbiol Mol Biol Rev., 74(3):417–33

[10]. Dabo, N.T., Muhammad, B., Saka, H.K., Kalgo, Z.M. & Raheem, R.A. (2019). Antibiotic Resistance Pattern of Escherichia coli Isolated from Diarrhoeic and Non-diarrhoeic Under Five Children in Kano, Nigeria. Journal of Microbiology and Biotechnology, 4(3):94–102.

[11]. Donlan, R.M. (2002). Biofilms: microbial life on surfaces. Emerg Infect Dis.,8(9):881.

[12]. Flores-Mireles, A.L., Walker, J.N., Caparon, M. & Hultgren, S.J. (2015). "Urinary tract infections: epidemiology, mechanisms of infection and treatment options," Nature ReviewsMicrobiology, 13(5):269–284 DOI 10.1038/nrmicro3432.

[13]. Kenreigh, C.A. & Wagner, L.T. (2006). Probiotic supplements ease diarrhea and other gastrointestinal disorders. Medscape Pharm., 12: 14-20. doi: 10.1089/act.2006.12.14

[14]. Mahsa, Y., Hedayat, H., Sedigheh, M., Elham, S. T. & Seyed, M. Z. (2017). Antibiofilm Effects of Lactobacilli against Ciprofloxacin-Resistant Uropathogenic Escherichia coli strains in Pasteurized Milk. Applied Food Biotechnology, 4 (4):241-250.

[15]. Mejlholm, O. and Dalgaard, P. (2015). Modelling and predicting the simultaneous growth of Listeria monocytogenes and psychrotolerant lactic acid bacteria in processed seafood and mayonnaise-based seafood salads. Food Microbiology 46: 1–14.

[16]. Nandakumar, V., Chittaranjan, S., Kurian, V.M. & Doble, M. (2013). Characteristics of bacterial biofilm associated with implant material in clinical practice. Polym J., 45(2):137.

[17]. Neupane, S., Pant, N.D., Khatiwada, S., Chaudhary, R. & Banjara, M.R. (2016). Correlation between biofilm formation and resistance toward different commonly used antibiotics along with extended spectrum beta lactamase production in uropathogenic Escherichia coli isolated from the patients suspected of urinary tract infections visit. Antimicrob Resist Infect Control, 5(1):5.

[18]. Osungunna, M.O. &Onawunmi, G.O. (2018). Antibiotic resistance profiles of biofilm-forming bacteria associated with urine and urinary catheters in a tertiary hospital in Ile-Ife, Nigeria. Southern African Journal of Infectious Diseases, 33(3):80–5.

[19]. Rafique, M., Potter, R.F., Ferreiro, A., Wallace, M.A., Rahim, A., Ali, M. A., Siddique, N., Abbas, M.A., D'Souza, A.W., Burnham, C.A.D., Ali, N. and Dantas, G. (2020) Genomic Characterization of Antibiotic Resistant Escherichia coli Isolated from Domestic Chickens in Pakistan. Front. Microbiol. 10:3052. doi: 10.3389/fmicb.2019.03052

[20]. Rao, K.P., Chennappa, G., Suraj, U., Nagaraja, H., Raj, C.A.P. & Sreenivasa, M.Y. (2015). Probiotic potential of Lactobacillus strains isolated from sorghum-based traditional fermented food. Probiotics Antimicrob Proteins., 7(2): 146- 156. doi: 10.1007/s12602-015-9186-6.

[21]. Soleimani, N.A., Kermanshahi, R.K., Yakhchali, B. & Sattari, T.N. (2010). Antagonistic activity of probiotic lactobacilli against Staphylococcus aureus isolated from bovine mastitis. Afr J Microbiol Res., 4(20): 2169-2173.

[22]. Syukur, S., Fachrial, E. and Jamsari. (2014). Isolation, Antimicrobial activity and protein bacteriocin characterization of lactic acid bacteria isolated from dadih in Solok, West Sumatera, Indonesia. Research Journal of Pharmaceutical, Biological and Chemical Sciences 5 (6): 1096–1104.

[23]. Tenaillon, O., Skurnik, D., Picard. B. & Denamur, E. (2010). The population genetics of commensal Escherichia coli. Nature Reviews. Microbiology 8 (3): 207–217.

[24]. Wiles, T.J., Kulesus, R.R. & Mulvey, M.A. (2008). Origins and virulence mechanisms of uropathogenic Escherichia coli. Exp Mol Pathol., 85(14)156-164

[25]. Yi-Te, C., Shigemura, K., Nishimoto, K., Yamada, N., Kitagawa, K., Sung, S.Y., Chen, K.C. & Fujisawa, M. (2020). Urinary tract infection pathogens and antimicrobial susceptibilities in Kobe, Japan and Taipei, Taiwan: An international analysis. J. Int. Med. Res., 48.

[26]. Zhu, M., Burman, W.J., Jaresko, G.S., Berning, S.E., Jelliffe, R.W. & Peloquin, C.A. (2001). Population pharmacokinetics of intravenous and intramuscular streptomycin in patients with tuberculosis. Pharmacotherapy, 21(9):1037-45.

Contribution of Individual Authors to the Creation of a Scientific Article (Ghostwriting Policy)

The author contributed in the present research, at all stages from the formulation of the problem to the final findings and solution.

Sources of Funding for Research Presented in a Scientific Article or Scientific Article Itself

No funding was received for conducting this study.

Conflict of Interest

The author has no conflict of interest to declare that is relevant to the content of this article.