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RESEARCH ARTICLE OPEN ACCESS

Antibacterial Activity of Cell-Free Supernatants of Probiotic *Lactobacillus* against Bacterial Pathogens Associated with Vaginal Infections

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Abstract

Vaginal infections are common female disease conditions that account for the prevalence of gynecological disorders which facilitate the increasing antimicrobial resistance and failure of prevalent treatment choices. In this study, the antibacterial activity of cell free supernatants (CFS) of probiotic *Lactobacillus* obtained from ogi (fermented maize) was evaluated against bacterial pathogens associated with vaginal infections. Bacterial pathogens isolated from high vaginal (n=22) and endocervical swabs (n=18) were bio-typed and assayed for hemolytic activity, biofilm production, antibacterial susceptibility pattern, and the CFS antagonistic activity. The occurrence of the vaginal bacterial pathogens was 33.0% for *Streptococcus* spp. and 31.0% for *Staphylococcus aureus,* with more than 70% resistance rates to amoxicillin, cefotaxime, imipenem/cilastatin, nalidixic acid, nitrofurantoin, cefuroxime, ceftriaxone sulbactam, ampiclox, cefixime and levofloxacin. More than 30% of the isolates produced biofilms. Of the four identified probiotic strains, only CFS from *L. plantarum* and *L. acidophilus* exhibited observable antagonistic reaction, with *L. plantarum* showing higher antibacterial activity against *Staphylococcus condimenti,* and *L. acidophilus* against *Klebsiella pneumoniae.* With the results of this study revealing the antibacterial activity of probiotic *Lactobacillus* CFS against vaginal bacterial pathogens, probiotic *Lactobacillus* can be suggested for use as prophylactic and bioprotective agents in the therapeutic management of vaginal bacterial infections and preservation of the vaginal microbiota.

Keywords: Probiotics, Ogi, *Lactobacillus, Staphylococcus, Klebsiella,* Vaginal Pathogens

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INTRODUCTION

Diverse bacterial flora in the female genitalia are reported to have a significant impact on vaginal health and flora.[1] Vaginal infections are one of the most common female dysfunctions with severe morbid circumstances that account for the prevalence of gynecological disorders.[2] The prevalence of bacterial vaginosis (BV) exhibits significant variation across different countries, with reported rates ranging from 20% to 60%.[3] The presence of vaginal discharge accompanied by symptoms such as pruritus, erythema, and occasional dyspareunia affecting the vaginal and vulval regions arise from the impacts of bacterial pathogens.[4] In spite of the reported less fatality of vaginal infections, the huge economic loss and treatment downtime are of special concern.[5] Several risk factors predispose adult females mostly at reproductive age to floral imbalance that leads to high level vaginal morbidity. These risk factors include ethnicity, genetics, antibiotic use, hygiene, sexual activity, infections, hormones, lifestyle and food.[6] The reports of Chee et al.[7] highlight that changes in the microflora of the vagina have the potential to modify immune responses and promote the proliferation of pathogens. This facilitates the development of several diseases that promote major aerobic vaginitis such as *Escherichia coli, Streptococcus, Pseudomonas* spp. and *Staphylococcus aureus* reported most frequently in patients with aerobic vaginitis.[8]

Metronidazole or clindamycin are typically the preferred therapeutic agents to treat infections of the vaginal region.[9] The reports of Serwecinska[10] opine that excessive and uncontrolled administration of antibiotics can result in the emergence of antibiotic resistance, and consequently increasing secondary infections in patients. However, Prabhurajeshwar & Chandrakanth,[11] had suggested probiotics as essential natural, safe and less toxic antimicrobial options to be utilized as alternative therapy for the management of bacterial-related vaginal infections, due mainly to the reports of increasing resistance and failure of antimicrobial treatment choices. *Lactobacillus* species, generally regarded as safe and frequently utilised as probiotics, are the largest genus among the lactic acid bacteria (LAB).[12,13] More so, they possess the potential to improve vaginal infection conditions due to their ability to generate antibacterial agents.[14] Literature has highlighted two distinct approaches for the administration of probiotic lactobacilli - oral and intravaginal.[15,16] Orally administered probiotic lactobacilli undergo a sequential transit through the oral cavity, stomach, intestines, and colon, before ultimately reaching the vaginal region through cutaneous contact in the perineal area. The delivery of probiotics to the vaginal region typically occurs within a timeframe of approximately seven days.[17] The intravaginal administration of probiotic lactobacilli can be facilitated through the utilisation of external equipment (an applicator). Thereafter, the effects of *lactobacillus* strains become evident within a period of 2-3 days.[18] The composition of lactic acid, antimicrobial peptides and bacteriocins in probiotics cell free supernatants (CFS) are potent components enforcing the antagonistic outcomes on pathogenic bacteria,[19] thereby inhibiting their replication and maintaining the vaginal ecosystem. Therefore, the study is aimed at evaluating the antibacterial activity of probiotic *Lactobacillus* against bacterial pathogens associated with vaginal infections obtained at the Federal Medical Center, Abeokuta, Nigeria.

METHODOLOGY

Ethical Approval

Ethical approval was obtained from the Covenant Health Research Ethics Committee (CHREC), with each study participant providing written informed consent that was kept confidential.

Selection of Subjects and sample collection

The exclusion criteria applied in the selection of the participants include pregnant and postpartum women, individuals with immunosuppressive conditions, women that have reached menopausal stage and subjects with recent history of antibiotic use within the last three (3) weeks. Vaginal swab samples including high vaginal (n=22) and endocervical (n=18) samples were collected from individuals already diagnosed for vaginosis, aged between 20-45 years, and attending the out- and in-patients' clinics of the

Federal Medical Center, Abeokuta, Nigeria; for therapeutic management.

Bio-typing, hemolytic pattern and biofilm production assay

Aliquots of the collected swab samples were inoculated on Blood and MacConkey Agars, and incubated for 24 h at 37°C. The isolates were characterized using the Analytical Profile Index for Staphylococci (API 20S) and the Analytical Profile Index for Enterobacteriaceae (API 20E) (bioMérieux, Inc, Durham, USA). The hemolytic activity of the strains was tested by inoculation of pure strains onto 7% Blood Agar, and incubated for 24 h at 37°C, and the pattern of lysis interpreted according to Thakkar et al.[20] Biofilm production of the strains was determined using the micro-bioassay method as described by Akinduti et al.[21] in a sterile 96-well microtiter plates. Aliquots (200 µL) of freshly prepared 0.5 McFarland Nutrient Broth containing the bacterial isolates were dispensed into each well of the microtiter plate and incubated for 24 h at 37°C. After incubation, the microtiter plate was rinsed twice with water to discard non-adherent cells, before introduction of 50 µL of crystal violet into all the wells and allowed to stand for 2 minutes. Following another round of rinsing, 100 µl of ethanol was added to each stained well, before evaluating the absorbance of the developed color intensity using a UV spectrophotometer at 630nm. The level of biofilm production was determined according to Stepanovic et al.[22]

Antibiotics Susceptibility Assay of Vaginal Bacterial Pathogens

Susceptibility of each bacterial strain to different antibiotics was evaluated using agar diffusion method according to the method of Dragomirescu et al.[23] These included amoxicillin (30 µg), cefotaxime (25 µg), cefixime (5 µg), imipenem/cilastatin (10/10 µg), nalidixic acid (30 µg), ofloxacin (5 µg), gentamycin (10 µg), nitrofurantoin (300 µg), cefuroxime (30 µg), ceftriaxone sulbactam (45 µg), ampiclox (10 µg) and levofloxacin (5 µg). Each bacterial isolate was diluted to 0.5 McFarland standard, before evenly spreading on Muller Hinton agar plate to make a lawn culture using a sterile swab stick. The plates were allowed to dry before placing the antibiotics impregnated disks, and incubating at 37 °C for 24 h. Thereafter, the areas of inhibition were estimated values interpreted according to the CLSI, 2020 guidelines.[24]

Isolation and Characterisation of *Lactobacillus*

Aliquots of serially diluted ogi (1g) were inoculated on De Man, Rogosa and Sharpe (MRS) agar and incubated for 48 h at 37°C under anaerobic condition.[25] The isolated strains were further characterized using preliminary test (such as catalase), carbohydrate fermentation test, and Analytical Profile Index for Lactobacilli (API 50 CHL).[26]

Probiotic Assay

Microtiter plates with 96 wells were used for the phenol, bile and acid tolerance assays. The selected LAB isolates were grown under anaerobic conditions in MRS Broth at 37°C overnight and 100 µl of diluted broth culture was added to bile salt solutions (0.1%, 0.3%, 0.7% and 1.0%), phenol solutions (0.1%, 0.3%, 0.4% and 1.0%), cholesterol (200mg), MRS broths of varying pH (2, 3, 4 and 5.5), respectively. A 100 µl of sterile MRS broth (as negative) and the selected LAB were also prepared. The absorbance of each solution was read at 630 nm at 0 and 6 h to determine the relative growth rate.[27]

Antibiotics Susceptibility Assay of *Lactobacillus*

Antibiotic susceptibility of the selected *Lactobacillus* with probiotic properties were evaluated with agar diffusion technique using various classes of antibiotics that include ceftazidime (30 µg), ciprofloxacin (5 µg), cefuroxime (30 µg), nitrofurantoin (300 µg), gentamicin (10 µg), augmentin (30 µg), cefixime (5 µg) and ofloxacin (5 µg). Each *Lactobacillus* strain was diluted to 0.5 McFarland standard, inoculated on to MRS agar plates and allowed to dry, before placing the antibiotics-impregnated disks. The plates were incubated at 37°C for 24 h under anaerobic conditions and the observed zones of inhibition estimated and interpreted according to the CLSI, 2020 guidelines.[24]

Antibacterial Activity of the CFS

Pure cultures of *Lactobacillus* were transferred into MRS broth and incubated at

37°C. After 48 hours incubation, the broth culture was centrifuged at 3,000 rpm for 15 minutes and filtered using a 0.22 µm sterile filter. The CFS was collected into sterile container for antibacterial analysis against the isolated vaginal bacteria pathogens using agar well diffusion technique reported by Reuben et al.[28] Overnight broth culture of the bacteria pathogen was adjusted to 0.5 McFarland and smoothly spread on nutrient agar plate. A sterile cork borer was used to make wells of 6 mm diameter and loaded with 50 µl of the CFS. To ensure a positive control, standard antibiotic, streptomycin (10 µg/ml) was added to one of the wells, then the plates were incubated for 24 h at 37°C. After incubation, the areas of inhibition were quantified and interpreted as resistant (0- 7 mm); susceptible (>10mm), as described by Kamble et al.[29]

Statistical Analysis

Significance of the antimicrobial activity of the probiotic LAB against the isolated vaginal pathogens was determined using chi-square and level of significance of the antimicrobial activity of the CFS was determined using ANOVA at p-value < 0.05. The statistical analyses were performed using SPSS 22.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Prevalence rate of bacteria pathogen associated with vagina infection

The vaginal swab cultures obtained from the women diagnosed (n=40) with vaginal bacterial infection, revealed a higher rate of *Streptococcus* spp. (33%), *S. aureus* (31%) and *E. coli* (13%) and very low rate of other strains including *Klebsiella* spp. (11%), *Pseudomonas* spp. (6%) and *Enterobacter* spp. (6%) as presented in Figure 1.

Antibiotics susceptibility pattern and biofilm production

The isolates exhibited 75% resistance rates to amoxicillin, cefotaxime, imipenem/

Table 1. Rate of biofilm formation among the strains

Bacteria Isolate	Biofilm Production n/N (%)
Staphylococcus aureus	6/17(35)
Escherichia coli	3/8 (37.5)
Enterobacter cloacae	1/3 (33)
Pseudomonas aeruginosa	0/6 (0)
Klebsiella pneumoniae	0/6 (0)
Streptococcus pneumoniae	1/18 (6)

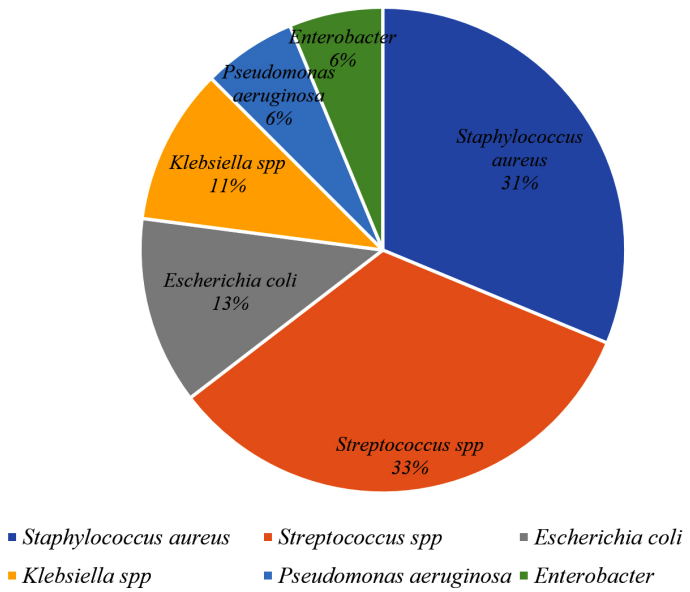

Figure 1. Distribution of biotypes from the vaginal infections

cilastatin, nalidixic acid, nitrofurantoin, cefuroxime, ceftriaxone sulbactam, ampiclox, cefixime and levofloxacin were recorded among the vaginal pathogens while significant susceptibility to levofloxacin and ofloxacin was observed (Figure 2). *E. coli, S. aureus* and *Enterobacter* spp. of more than 30% showed high biofilm production, as shown in Table 1.

Probiotic Assay on *Lactobacillus*

Among the nine *Lactobacillus* isolates isolated from the fermented ogi samples, only four isolates PL1, PL4, PL8 and PL9 showed more than 80% tolerance to acid and bile solution at pH 2 and 3, 0.3% bile concentration and 0.4% of phenol (Table 2). Further assessments revealed that the four selected strains of *Lactobacillus* exhibited no hemolytic activity, although susceptible to all the antibiotics tested (Table 3).

CFS Antibacterial Activity

The cell free supernatants of the selected probiotics strains assessed for antibacterial activity against isolated bacteria pathogens obtained from vaginal infections as highlighted in Table 4, showed varying zones of inhibition. Results show that CFS from *Lactobacillus plantarum* CFS exhibited good antibacterial activity against *Staphylococcus condimenti* (15mm), with *Lactobacillus acidophilus* showing significant zone of inhibition (12mm) against *Klebsiella pneumoniae*; while *L. brevis* and *L. fermentum* exhibited a diverse range of

Table 2. Probiotic survival assay

	Acid tolerance				Bile tolerance				Cholesterol	Phenol			
Isolate Codes	pH2	pH3	pH4	pH5.5	0.1%	0.3%	0.7%	1.0%	200mg	0.1%	0.3%	0.4	1.0
PL1	151.65	129.90	142.61	111.19	91.83	86.58	79.15	73.19	86.48	99.04	98.34	94.53	84.85
PL2	78.96	79.83	89.69	108.92	86.65	72.00	71.65	63.32	85.39	86.34	77.89	74.06	70.38
PL3	75.73	73.55	92.67	99.88	80.55	76.48	69.14	71.04	92.08	72.14	59.89	51.94	50.74
PL4	102.89	97.47	101.59	95.66	87.83	82.66	78.55	71.87	85.28	94.44	93.59	94.43	91.91
PL5	77.12	78.51	80.06	81.56	85.53	76.38	70.78	67.99	89.05	98.75	86.29	79.59	66.57
PL6	79.01	79.73	85.54	106.39	90.61	79.87	71.18	65.25	95.24	51.97	71.61	62.86	49.38
PL7	78.85	79.38	89.98	115.88	88.97	74.87	58.28	48.81	91.69	80.19	70.28	78.39	70.88
PL8	100.19	99.36	100	92.11	90.76	84.27	75.55	70.92	99.42	89.74	98.23	93.65	81.94
PL9	116.89	118.22	115.91	109.08	89.29	84.24	78.87	72.78	89.82	98.24	99.14	92.58	86.40

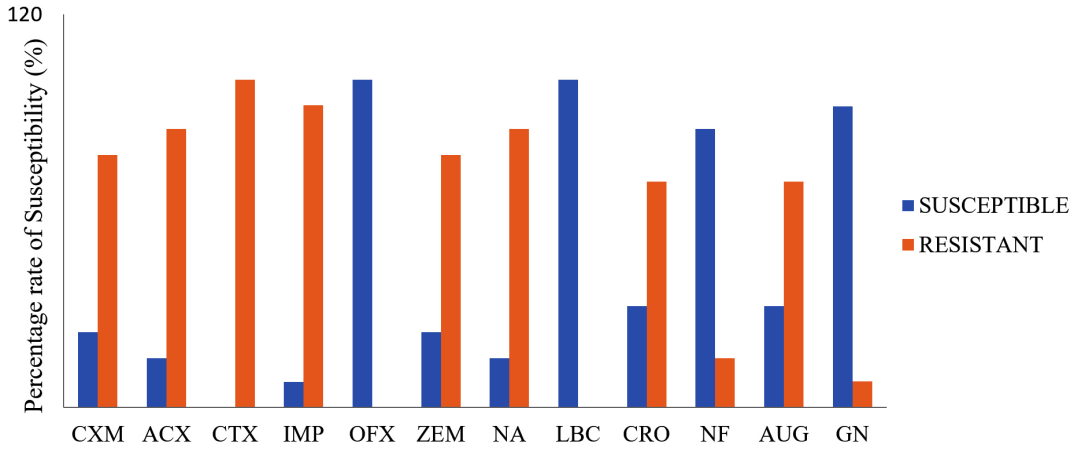

Figure 2. Susceptibility pattern of the vaginal bacterial strains to various classes of antibiotics
KEY: CXM- Cefuroxime, ACX- Ampiclox, CTX- Cefotaxime, IMP- Imipenem/Cilastatin, OFX- Ofloxacin, ZEM- Cefixime, NA- Nalidixic Acid, LBC- Levofloxacin, CRO- Ceftriaxone Sulbactam, NF- Nitrofurantoin, AUG- Amoxicilin Clavulanate, GN- Gentamycin

Table 3. Antibiotics susceptibility of selected probiotics

Antibiotics	Isolates			
	L. fermentum	*L. brevis*	*L. plantarum*	*L. acidophilus*
Ceftazidime	S	S	S	S
Ciprofloxacin	S	S	S	S
Cefuroxime	S	S	S	S
Nitrofurantoin	S	S	S	S
Gentamicin	S	S	S	S
Augmentin	S	S	S	S
Cefixime	S	S	S	S
Ofloxacin	S	S	S	S

Key: R for resistance; S for susceptible

Table 4. Antibacterial pattern of CFS from Probiotic LAB

API Identity	*S. aureus*	*K. pneumoniae*	*P. aeruginosa*	*S. condimenti*	*E. cloacae*	*E. coli*	*P. values*
	Zones of inhibition (mm)						
L. fermentum	11	11	10	10	9	8.5	0.055
L. brevis	10	11	10	11	9	8.5	0.034
L. plantarum	10	11	10	15	11	8.5	0.02
L. acidophilus	9	12	11	11	9	11	0.02

antibacterial activity against the vaginal pathogens. *L. plantarum* and *L. acidophilus* exhibited the most significant zone of inhibition compared to *L. brevis* and *L. fermentum.*

DISCUSSION

The decrease in essential Lactobacilli residing in the vaginal region, alongside the possible increased presence of pathogenic bacteria represents a noteworthy determinant in the potential onset of vaginal infections. The vaginal microflora of individuals with vaginal infections is primarily inhabited by various pathogenic microorganisms that may include bacteria of the *Streptococcus, Staphylococcus, E. coli,* and *Enterobacter* genera. A vaginal microbiome that is in equilibrium has the capacity to present protection against vaginal infections. In this study, *Lactobacillus fermentum, Lactobacillus plantarum, Lactobacillus brevis* and *Lactobacillus acidophilus* showed susceptibility to all the tested antibiotics. This makes these strains safe because they do not possess resistant genes that could be transferred to the vagina pathogens.[30] Also, the reports of Mohankumar et al.[31] suggest that the restorative therapy of vaginal microbiota appears to be negatively affected by the susceptibility of *Lactobacillus* strains to antibiotics.

The current investigation reports that the *Lactobacillus* obtained from fermented ogi demonstrated probiotic characteristics and agrees with the reports of Olatunde et al.,[32] who documented the isolation of probiotic *Lactobacillus* from waste products obtained during ogi manufacturing. More so, the *Lactobacillus* exhibited antibacterial efficacy against the pathogenic bacteria strains from patients diagnosed with vaginal infections. The results of this study aligns with numerous prior studies that have demonstrated the capacity of *Lactobacillus* to generate organic acids, hydrogen peroxide and bacteriocins among others.[2] Since four *Lactobacillus* strains isolated from fermented ogi demonstrated probiotic properties, it could be inferred that fermented ogi possesses adequate probiotic LAB that could be harnessed for both antimicrobial activity and source, with high survival rates above 80% in the gastrointestinal

tract (GIT). This feature could be a crucial factor in promoting the use of oral probiotics. In this context, it is imperative that probiotics possess non-pathogenic properties, exhibit resilience in the midst of unfavorable conditions present within the gastrointestinal tract and ultimately arrive at the intestine in an efficient/viable state.[33]

Furthermore, it was observed that the CFSs of the four *Lactobacillus* strains exhibited inhibitory effects on the vaginal pathogens, showing a varying spectrum of activity. Dasari et al.[2] has previously demonstrated the antibacterial efficacy of the bacteriocin derived from the vaginal probiotic *Lactobacillus* against a range of pathogens that include *Staphylococcus aureus, Pseudomonas aeruginosa, Enterobacter cloacae, Escherichia coli, K. pneumonia,* and *Streptococcus pneumonia* isolated from individuals with cervicovaginal infections. More so, Andreeva et al.[34] has reported that CFS from *Lactobacillus* strain exhibited antibacterial properties against isolated vaginal pathogen. In a study conducted by Faniran & Omemu,[35] probiotic LAB CFS demonstrated the ability to inhibit the proliferation of *S. aureus, Proteus mirabilis, Escherichia coli, Bacillus cereus* and *K. pneumonia.* The significant antimicrobial activity of the probiotic CFS revealed the potential of organic acids, hydrogen peroxide and bacteriocins in *Lactobacillus*-dominant environments to provide protection to the host against infections caused by pathogenic organisms. This is achieved through the synthesis of antimicrobial substances and short chain fatty acids, which serve to acidify the local microenvironment and maintain vaginal pH levels below 4.5.[36] Some examples of the metabolites that have been shown to have a positive effect on vaginal health include bacteriocin, lactic acid, hydrogen peroxide and acetic acid among others.[37-39]

Researchers have found that hydrogen peroxide produced by *Lactobacillus* is beneficial against sexually transmitted diseases and bacterial vaginosis.[40] The breakdown products of glycogen are used in the generation of lactic acid under anaerobic conditions, resulting in a decrease in pH of the vagina.[17,41] This acidification acts as a physiological defense mechanism that can potentially boost the efficacy of other immunomodulatory and antibacterial capabilities, by penetrating cell membranes and producing osmotic stress, as well as by destabilizing the outer membrane of vaginal pathogenic Gram-negative organisms.[42]

CONCLUSION

This study demonstrated that CFS from probiotic *Lactobacillus* sourced from fermented ogi contains antimicrobial compounds that exhibit antibacterial activity against pathogens isolated from the vagina. Therefore, it could be plausible to utilize CFS from probiotic LAB as prophylactic and bioprotective agents for the management of vaginal bacterial infections, while also preserving the typical vaginal microbiota.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This study was approved by the Covenant Health Research Ethics Committee (CHREC), Covenant University, Ogun State, Nigeria, with reference number CU/HREC/EGN/206/23.

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