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# ANTAGONISTIC EFFECT OF FOUR *LACTOBACILLUS SP.* ON MULTIDRUG RESISTANT *KLEBSIELLA SP.* GF01 IN COCULTURE

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## ABSTRACT

The global antibiotic resistance in uropathogens has resulted in a search for alternatives to antibiotics. Lactic acid bacteria have a long history of safe use in the fight against various infectious states. This study is aimed at determining the antimicrobial activities of 4 species of lactobacilli previously isolated from Nigeria women during different stages of menstrual cycle against multidrug resistant uropathogenic *Klebsiella* sp. GF01 in coculture.
Four LAB has been previously identified from Nigerian women by sequencing the 16S rRNA gene. The antimicrobial properties of the LAB were tested against multidrug resistant uropathogenic strain of *Klebsiella* sp. GF01 using coculturing. *Klebsiella* sp. GF01 was freshly and at 8 hours growth, introduced into overnight growth of each lactobacilli, incubated for 24 hours, after which bacterial count of the uropathogen and *Lactobacillus* strains were performed In both series of experiment, *Lactobacillus brevis* GF021, *Lactobacillus fermentum* GF002 and *Lactobacillus plantarum* GF011 were active on *Klebsiella* sp. GF01 with $6\log_{10}$ reduction ($10^{10}$cfu/ml-$10^{4}$cfu/ml) after 24 h, while *Lactobacillus fermentum* GF019 show a low activity on *Klebsiella* sp. GF01 with $2\log_{10}$ and $4\log_{10}$ reduction ($10^{10}$cfu/ml-$10^{8}$cfu/ml and $10^{10}$ cfu/ml-$10^{6}$cfu/ml) after 24 h for the first and second series of experiment respectively. It was observed that *Klebsiella* sp. GF01 do not have significant effect on any of the LAB strains. The presence of LAB was capable of inhibiting the growth of *Klebsiella* sp. GF01 freshly introduced and when it has already grown for 8h. The decrease in the number of *Klebsiella* sp. GF01 reveals the antagonistic effect of LAB cells on multidrug resistant Klebsiella sp. GF01.

**KEYWORDS**:

## INTRODUCTION

Lactic acid bacteria are the dominant microflora of the vagina of a normal healthy premenopausal woman [1] and they also maintains the typical acidic pH value of the vaginal environment (approximately pH 3.8 - 4.4) by secreting lactic acid [2]. This acidic pH value acts as a vaginal barrier, protecting it from pathogens. As a result, the healthy vagina can defend itself against infections [3]. The primary colonizing bacteria of a healthy individual are of the genus *lactobacillus* [4] such as *L. crispatus, L. acidophilus, L. bifidus, L. rhamnosus, L. fermenti, L. plantarum*, *L. brevis* etc . [5].
The ability of these *Lactobacilli* to serve as prophylactic or biotherapeutic agents can be attributed to various properties which they possess. One of these is their ability to adhere to and colonize tissues; another is the capacity to inhibit the pathogenesis of disease causing organisms. They also produce biosurfactants and several anti-adhesion molecules which inhibit the attachment and colonization of a broad range of pathogens [6]. Other properties include the ability to produce inhibitory substances such as hydrogen peroxide, organic acids and bacteriocins which are believed to be important in vaginal colonization [7]. The production of organic acid acts by reducing the pH of the environment and this may result in a decrease in survival time of a bacterial uropathogen. Bacteriocins which play considerable roles in regulation of pathogenic numbers in an environment and food also act by inhibiting the growth of pathogens by competition when present together in an environment. Ayeni and Adeniyi [8] had reported that the lactic acid bacteria belonging to the genus

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*Enteroccocus* and *Streptococcus* were isolated during menstruation.

Urogenital infection is characterized by fluctuation of the vagina flora from predominant of LAB to uropathogens as a result of hormonal fluctuation, sexual activities, diet and other factors [8]. *Escherichia coli* is the causative agent in most cases (up to 85%) followed by *Staphylococcus saprophyticus*, *Klebsiella pneumoniae* and enterococci [9]. Women are more prone to urinary tract infection than men because, in females, the urethra is much shorter and closer to the anus and as a woman's oestrogen levels decrease with menopause, her risk of urinary tract infections increases due to the loss of protective vaginal flora [10].. Bacterial isolates from UTI cases are usually characterized by steady increase in their level of resistance to commonly used antimicrobials including ampicillin, trimethoprim-sulphamethoxazole (TMP-SMX) or co-trimoxazole and the quinolones with most uropathogens being multidrug resistant [11]. Therefore, there is need for other alternatives for prevention and treatment of urinary tract infections especially in women.

Four *Lactobacilli* strains have earlier been isolated from Nigerian women during different stages of menstrual cycle. *L. brevis* GF021 (menstruation period), *L. plantarum* GF011 and *L. fermentum* GF002 (safe period) and *L. fermentum* GF019 (ovulation period) in our laboratory. The good antimicrobial activities of these strains which have previously been observed using the cell free supernatant and viable cells have call for further studies on the ability of the LAB to further reduce the growth of uropathogenic *Klebsiella* sp. in co culture. Therefore this recent study is aimed to investigate the ability of these four species of *Lactobacilli* in reducing the count of uropathogenic K*lebsiella* sp. in coculture.

## Materials and Method

### *Microorganisms*

For this study, four *Lactobacilli* strains which we have earlier isolated in our laboratory from Nigerian women during different stages of menstrual cycle and identified by sequencing the 16S rRNA gene were used. They are: *Lactobacillus brevis* GF021 (menstruation period), *Lactobacillus plantarum* GF011 and *Lactobacillus fermentum* GF002 (safe period) and *Lactobacillus fermentum* GF019 (ovulation period). The tested uropathogenic *Klebsiella* sp. GF01 was obtained from Medical Microbiology Unit of University College Hospital (UCH), Ibadan and further identified by standard biochemical tests (indole and citrate test). *Lactobacilli* strains were grown in de Mann Ragosa Sharpe (MRS) broth, incubated microaerophilically at 37$^{o}$C for 24 hours. *Klebsiella* sp. GF01 was cultured in Nutrient Broth, incubated aerobically at 37$^{o}$C for 24 hours

### Antibiotic Susceptibility Test of *Klebsiella* sp.

Three uropathogenic *Klebsiella* sp. were screened against seven antibiotics by disk diffusion methods. Twenty millimeter of Mueller Hinton agar was poured into a sterile petri dish and allowed to set. A bacterial lawn was accomplished by spreading inoculum from $10^{8}$ dilution factor of the pathogen culture which is approximately equivalent to 0.5 McFarland standards by a sterile swab stick. The antibiotic disks containing ceftazidime (30µg), cefotaxime (30µg), Cefuroxime (30µg), Augmentin (10µg), Ciprofloxacin (30µg), Ofloxacin (30µg), and Gentamycin (30µg) were placed firmly on the surface of the solidified agar with the aid of sterile forceps and incubated aerobically at 37$^{o}$C for 24 h. The susceptibility of the test organisms to the used antibiotics was evident by measuring the diameter of the clear zones of inhibition in millimeter (mm) around the antibiotics disks and the results were interpreted according to the guidelines of European Committee on Antimicrobial Susceptibility Testing [12]. The resistant strain was selected for antimicrobial study with lactobacilli.

### *Coculture growth*

The interference of LAB strains with the growth of uropathogenic *Klebsiella* sp. strain was evaluated by coincubating *Klebsiella* sp. GF01 with four LAB (*Lactobacillus brevis* GF021, *Lactobacilus fermentum* GF002, *Lactobacillus plantarum* GF011 and *Lactobacillus fermentum* GF019, This was done in two series of experiment.

In the first experiment, *Klebsiella* sp. GF01 was inoculated into overnight culture of grown LAB. 1 ml of overnight culture of *Klebsiella* sp. GF01 was inoculated into 5 ml double strength nutrient broth mixed together and then added to 5 ml overnight culture of LAB and incubated for 24 h. The monoculture of the LAB and *Klebsiella* sp. GF01 (control) was evaluated at time zero ($t_o$) by plating each LAB from an appropriate dilution factor onto MRS agar and incubated microaerophilically at 37$^{o}$C for 24 h and the *Klebsiella* sp. GF01 on MacConkey agar, followed by incubating aerobically at 37$^{o}$C for 24 h. After 24 h of coculture of LAB and *Klebsiella* sp. GF01, the mixture was serially diluted and

appropriate dilution factor of was plated accordingly to evaluate the growth of LAB and uropathogen.
For the second experiment, both *Klebsiella* sp. and the LAB were grown for 24 h and overnight culture of *Klebsiella* sp. GF01 was innoculated in a 5 ml of fresh nutrient broth and incubated for 8 h, after which it was centrifuged and the supernatant discarded, 5 ml of double strength nutrient broth was added to resuspend the pellets and added to a 5 ml of overnight culture of LAB making the whole mixture 10 ml. Serial dilution of both the *Klebsiella* sp. GF01 alone and the mixture was done at 8 h (which is 0 hr for mixture) and 24h, followed by plating the *Klebsiella* sp. GF01 as mentioned above during both hours to evaluate the growth of the *Klebsiella* sp. GF01.

## RESULTS

Antibiotic susceptibility test was done on three *Klebsiella* sp. strains. The three organisms used show a high resistance towards most of the antibiotics used (data not shown). *Klebsiella* sp. GF01 was selected because it showed 100% resistance to all tested antibiotics, i.e. 0 mm zones of inhibition to ceftazidime, cefotaxime, cefuroxime, augmentin, ciprofloxacin, ofloxacin and gentamicin.
The capability of four representatives LAB strains (*L. brevis* GF021, *L. fermentum* GF002, *L. plantarum* GF011 and *L. fermentum* GF019) to reduce the in vitro growth of *Klebsiella* sp. GF01 was evaluated in 2 coculture experiments. In both series of experiment, *Lactobacillus brevis* GF021, *L. fermentum* GF002 and *L. plantarum* GF011 were active on *Klebsiella* sp. GF01 with $6\log_{10}$ reduction (i.e $10^{10}$cfu/ml-$10^{4}$cfu/ml) after 24 h, while *L. fermentum* GF019 show a low activity on *Klebsiella* sp. GF01 with $2\log_{10}$ and $4\log_{10}$ reduction (i.e. $10^{10}$cfu/ml-$10^{8}$cfu/ml and $10^{10}$cfu/ml-$10^{6}$cfu/ml) after 24 h for the first and second series of experiment respectively. It was observed that *Klebsiella* sp. GF01 do not have significant effect on any of the LAB strains.

Due to the above data, it was observed that among the four LAB strains, *L. fermentum* GF019 from ovulation showed the least activity ($\leq 4\log_{10}$ reduction) and *L. brevis* GF021 show the highest activity ($6\log_{10}$ reduction) on *Klebsiella* sp. GF01. The presence of grown LAB was capable of inhibiting the growth of *Klebsiella* sp. GF01 freshly introduced and when it has already grown for 8h. The decrease in the number of *Klebsiella* sp. GF01 reveals the antagonistic effect of LAB cells on *Klebsiella* sp. GF01.

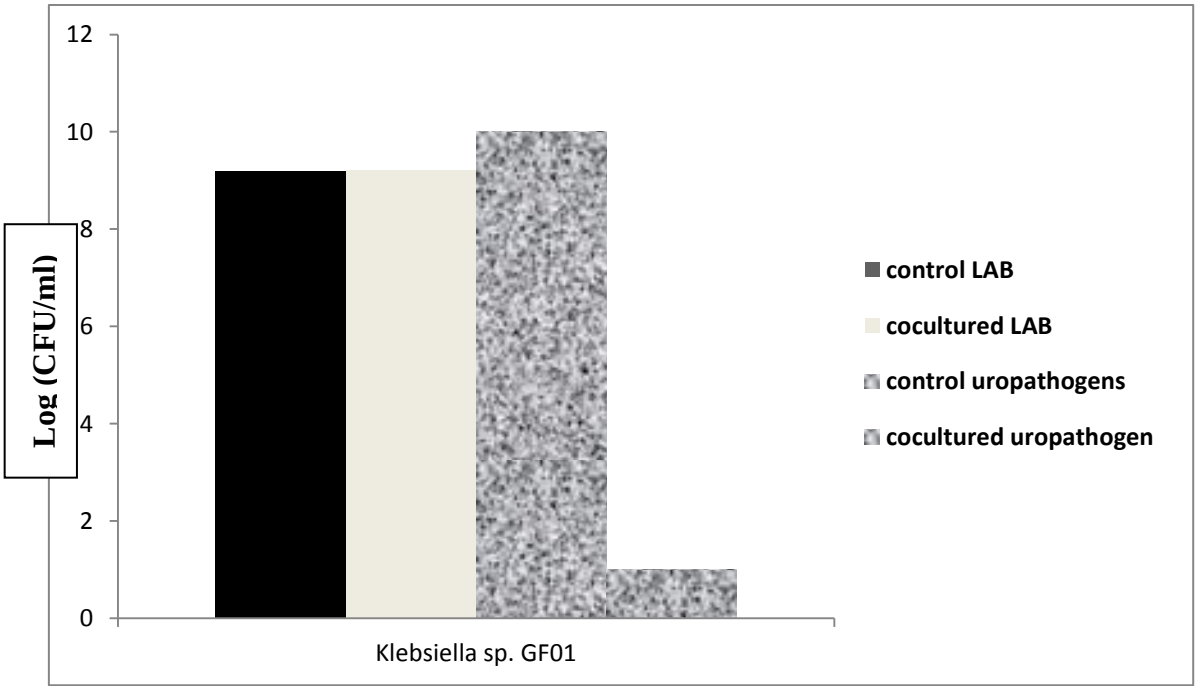


**Figure 1: Inhibition of in vitro growth of *Klebsiella* sp. GF01 by *L. brevis* GF021 after 24 hours co-incubation.**

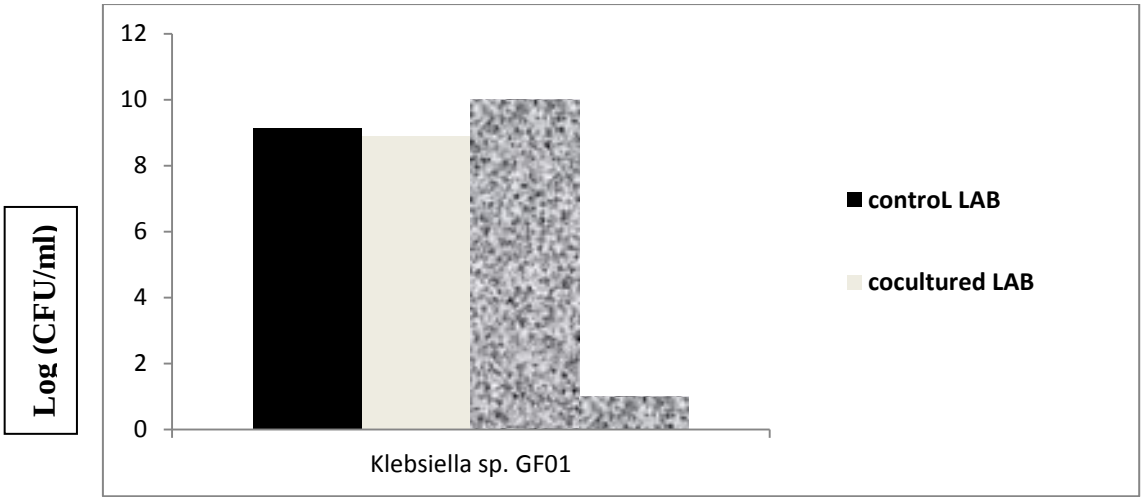


**Figure 2 Inhibition of in vitro growth of *Klebsiella* sp. GF01 by *L. fermentum* GF002 after 24 h co-incubation.**

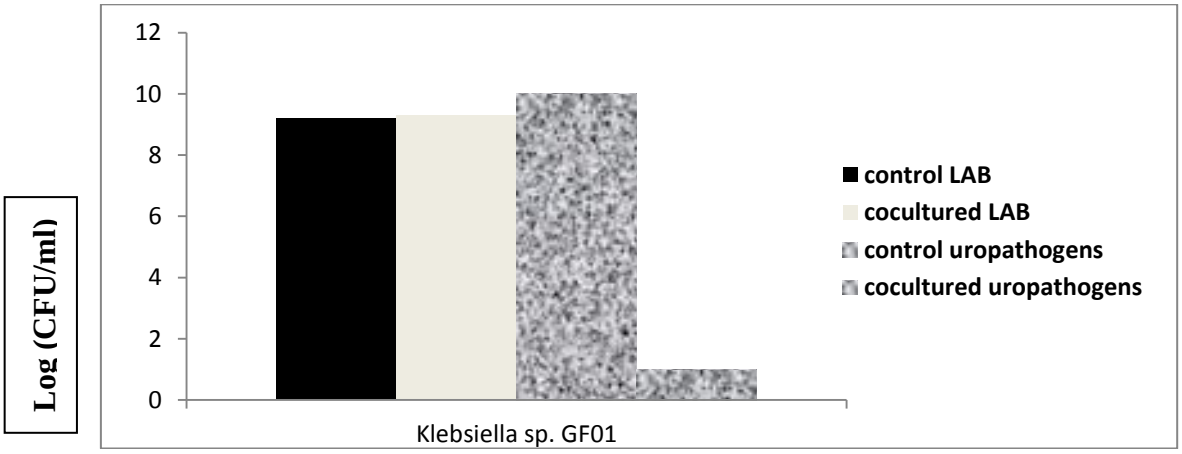


**Figure 3: Inhibition of in vitro growth of *Klebsiella* sp. GF01 by *L. plantarum* GF011 after 24 hours co-incubation.**

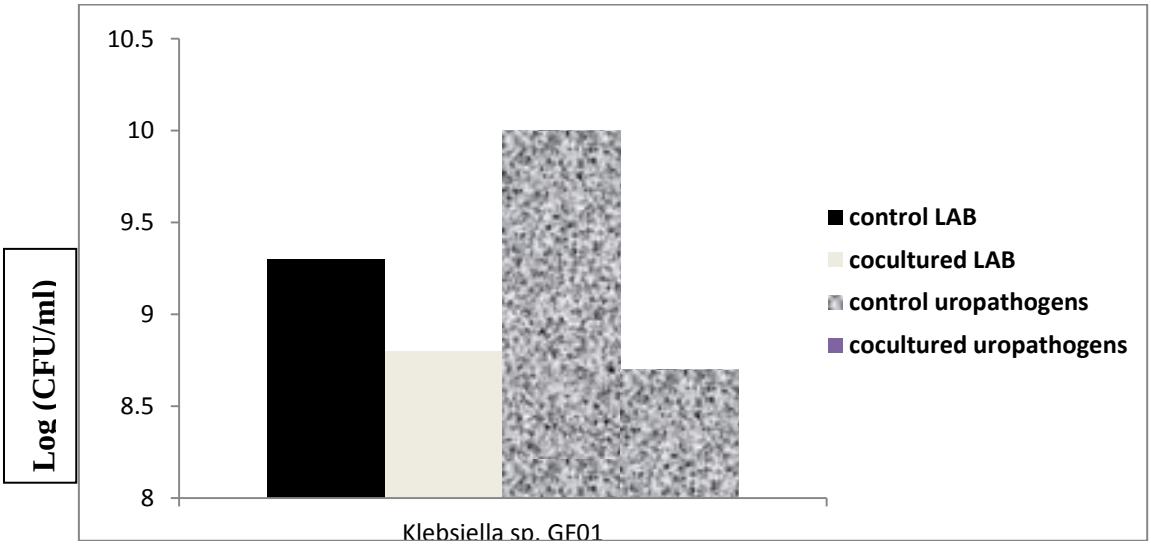


**Figure 4: Inhibition of in vitro growth of *Klebsiella* sp. GF01 by *L. fermentum* GF019 after 24 h co-incubation.**

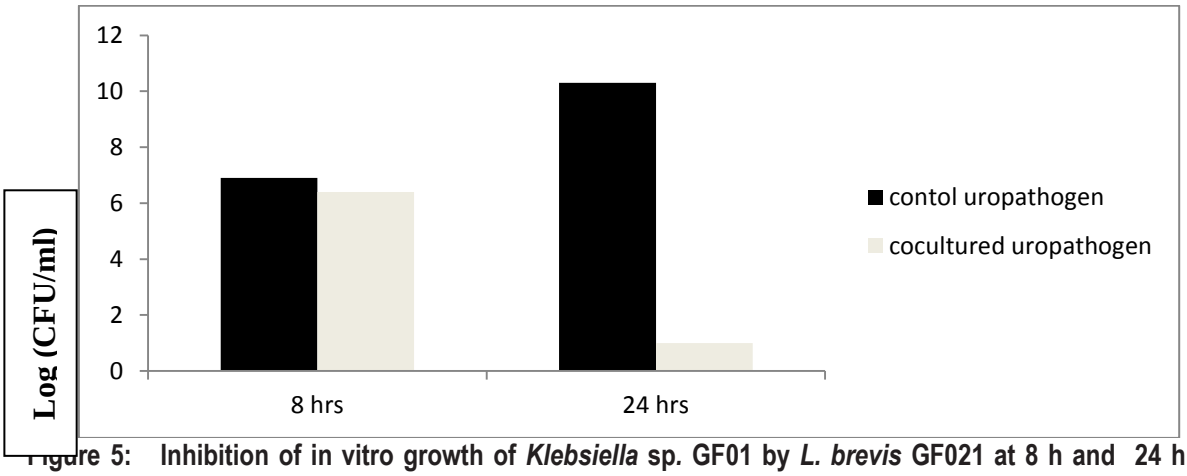


**Figure 5: Inhibition of in vitro growth of *Klebsiella* sp. GF01 by *L. brevis* GF021 at 8 h and 24 h co-incubation.**

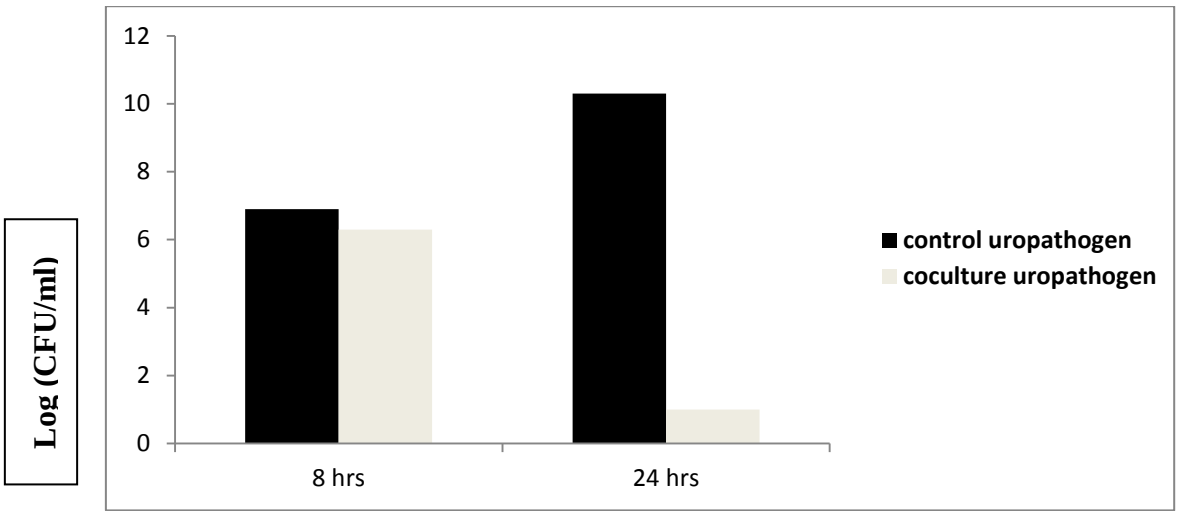


**Figure 6: Inhibition of in vitro growth of *Klebsiella* sp. GF01 by *L. fermentum* GF002 at 8 h and 24 h co-incubation**

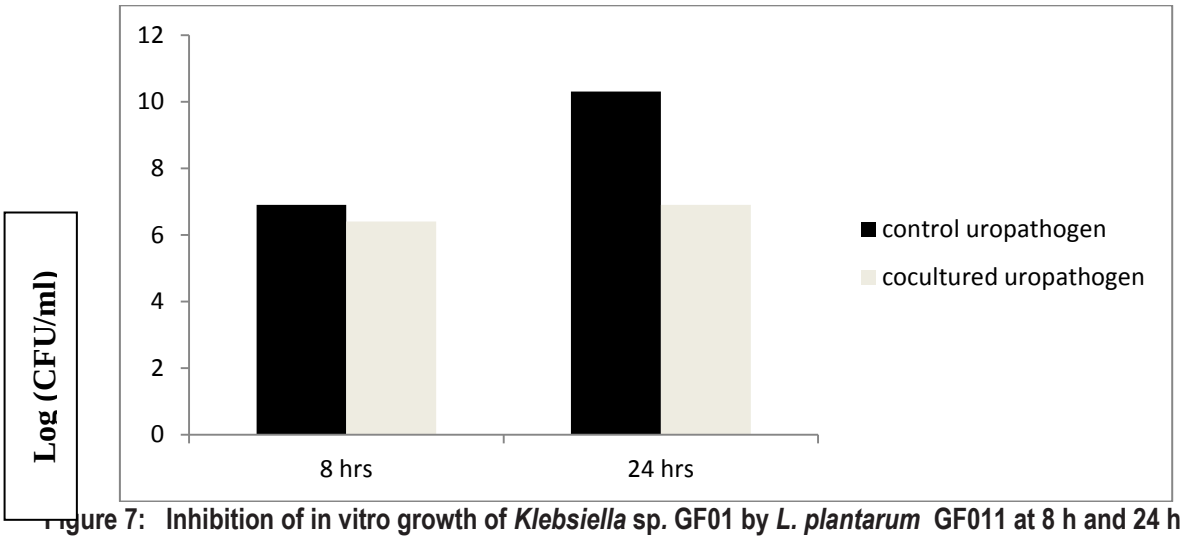


**Figure 7: Inhibition of in vitro growth of *Klebsiella* sp. GF01 by *L. plantarum* GF011 at 8 h and 24 h co-incubation.**

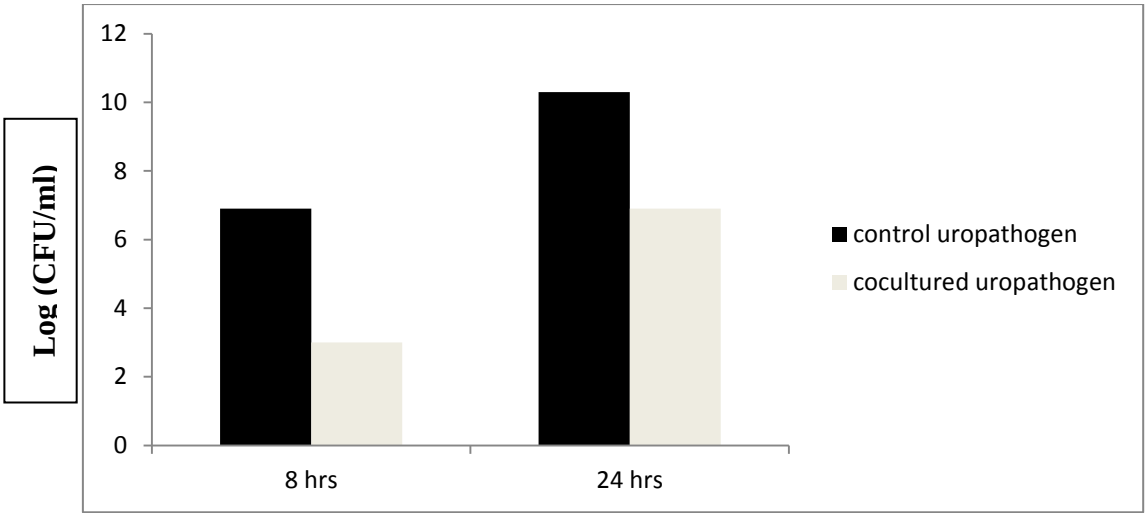


**Figure 8: Inhibition of in vitro growth of *Klebsiella* sp. *GF01* by *L. fermentum* GF019 at 8 h and 24 h co-incubation**

## DISCUSSION

In this study, uropathogenic *Klebsiella* sp. GF01 was found to be completely resistance to all tested antibiotics. This is alarming because *Klebsiella* sp. is the second most frequently isolated pathogen from UTI cases [13]. Reid and Seidenfeld [14] reported that drug resistance to commonly used antibiotics is increasing among uropathogens. The above discussed resistance pattern exhibited by pathogens responsible for community acquired urinary tract infections is similar to the resistance pattern also observed all over the world [11, 15]. Therefore, resistance of pathogens to antibiotics is a global problem and alternative prophylaxis and treatment for urinary tract infection is urgently needed. This is one of the reasons more people all over the world are accepting the concept of probiotics as alternative prophylaxis and treatment for urinary tract infection and hence, the main objective of this research work.

In this present study, it was shown that the *lactobacillus* strains which was previously been isolated from Nigerian women vaginal during different stages of menstrual cycle effectively reduce the growth of *Klebsiella* sp, GF01 either when inoculated freshly or when it has already grown for 8 h. In contrast, in both cases, the growth of the *Lactobacilli* strains was not greatly influenced by the presence of *Klebsiella* sp, GF01. During 24 h coculture, the strongest antagonistic activity was shown by *L, brevis* GF021 gotten from menstruation period, *L. fermentum* GF002 and *L, plantarum* GF011 gotten from safe period, which led to the 6 log reduction of the *Klebsiella* sp, GF01. *L fermentum* GF019 gotten from ovulation, which exhibit the weakest inhibition effect gives a ≤4 log reduction on *Klebsiella* sp, GF01, similar result was obtained by Renzi *et al*., [16] where it was reported that in the mixed population of *Lactobacillus plantarum* BG25 and *Klebsiella pneumoniae*, the cells of *Lactobacillus plantarum* BG25 retained high activity, and its concentration of viable cells increase, irrespective of the presence of pathogens, while that of *Klebsiella pneumoniae* was reduced at 24 h and at the 48th hour no viable pathogen cells were reported. The study of Lorenzo *et al.* [17] revealed similar process i.e coculture, to determine the antagonistic activity of different LAB strains against *E.coli* and *S.enteritidis*, where he reported an effective inhibition when inoculated simultaneously or when cultured overnight and then incubated with the pathogens

*L, brevis* and *L, plantarum* were reported by Beata *et al*., [18] to have completely inactivated *Salmonella sentenberg*. during 48 h of coculture but *L, brevis* and *L, plantarum* used in this study was able to reduce the growth of *Klebsiella sp*, GF01, Finally, the decrease in the number of *Klebsiella* sp. GF01 reveals the antagonistic effect of LAB cells on *Klebsiella* sp. GF01. The limited influence of pathogen on lactic acid bacteria strain was also reported by Lorenzo *et al*. [17].

The mechanisms by which all our strains reduce uropathogenic strains growth are not fully understood at present but the antagonistic activity of all these strains is probably due to the production of organic acid, which was reported by Alakomi *et al*., [19] to be responsible for the inhibition of Gram negative bacteria and other metabolites such as hydrogen peroxides and bacteriocins reported by Salminen *et al.,* [15].

It can be concluded that vaginal LAB strains isolated from healthy women in different stages of the menstrual cycle are able to inhibit the growth of *Klebsiella* sp. GF01 which is completely resistant to all tested antibiotics. It can be inferred that strains of vaginal LAB, can be used as alternative to antibiotics in management of multidrug resistant uropathogenic *Klebsiella* sp. Further studies are ongoing on the lactobacilli to test their further probiotic properties

## CONFLICT OF INTEREST

The authors have no conflict of interest in conducting and reporting this research

## REFERENCES


1. Coolen MJ, Post E, Davis CC, Forney LJ. Characterization of microbial communities found in the human vagina by analysis of terminal restriction fragment length polymorphisms of 16S rRNA genes. Appl. Environ. Microbiol. 71, 2005, 8729-8737.
2. Boskey ER, Telsch KM, Whaley KJ, Moench TR. Acid production by vaginal flora in vitro is consistent with the rate and extent of vaginal acidification. Infect. Immun. 67, 1999, 5170-5175.
3. Spence, D. Candidiasis (vulvovaginal), BMJ Clin Evid. 5, 2010, pii: 0815.
4. Vasquez A, Jakobsson T, Ahrne S, Forsum U, Molin G. Vaginal Lactobacillus Flora of Healthy Swedish Womem. Journal of Clinical Microbiology. 40 (8), 2002, 2746–2749
5. Shah NP. Functional cultures and health benefits, Int. Dairy J. **17**1, 2007, 262–1277.
6. Velreads MC, Vander-Mei HC, Reid G, Busscher HJ. Inhibition of initial adhesion of uropathogenic Enterococcus feacalis by biosurfactants from Lactobaccillusisolates. Appl. Enviro. Microbiol, 1996, 62:1958-1963
7. Agboola FM, Adeniyi BA, Okolo CA, Ogunbanwo TO, Ayeni FA, Lawal TO. Probiotic potentials of Lactic Acid Bacteria isolated from vaginal swabs on selected genital pathogens. IJPSR. 5(7), 2014, 2642-2650.
8. Ayeni FA, Adeniyi BA. Antimicrobial potentials of Lactic Acid Bacteria isolated from a Nigerian menstruating woman. TAF Prev. Med.Bull. 12 (3), 2013, 283-290
9. Stamm WE, Hooton TM. Management of urinary tract infections in adults. N. Engl. J. Med. 329, 1993, 1328-1334.
10. Dielubanza EJ, Schaeffer AJ. Urinary tract infections in women. The Medical clinics of North America. 95 (1), 2001, 27–41.
11. Garau J, Xercavins M, Rodriguez-Carballeria M, Gomez-Vera JR, Coll I, Vidal D. Emergency and dissemination of quinolones-resistant E. coli in the community. Antimicrob. Agents Chemother. 43, 1999, 2761–2841.
12. EUCAST - European Committee on Antimicrobial Susceptibility Testing. Enterobacteriaceae: Breakpoint tables for interpretation of MICs and zone diameters, Version 5.0, valid from 2015-01-01. http://www.eucast.org
13. Hamdan ZH, Kubbara E, Adam AM, Hassan OS, Suliman SO, Adam I. Urinary tract infections and antimicrobial sensitivity among diabetic patients at Khartoum, Sudan. Ann Clin Microbiol Antimicrob. 14, 2015, 26.
15. Reid, G, Seidenfeld A. Drug resistance amongst uropathogens isolated from women in a suburban population: J Uro, 1(4), 1997,432–7.
16. Salminen S, Isolauri E, Salminen E. Clinical uses of probiotics for stabilizing the gut mucosal barrier: Successful strains and future challenges, Antonie van Leeuwenhoek 1996, 70347–358
17. Remzi C, Yanakieva V, Denkova Z, Chakarova N. Antimicrobial activity of Lactobacillus plantarum BG25 against pathogenic microorganisms. научни трудове на русенския университет. 53, 2014, 10.2
18. Lorenzo D, Gismondo R, Alessandra L, Christoph H, Luigia G. Inhibition of invitro growth of enteropathogens by new lactobacillus isolates of human intestinal origin. FEMS Microbiology letters. 153, 1997, 455-463
19. Beata S, Zbigniew P, Ilona M. Antagonistic effect of Lactic Acid Bacteria on Samonella senftenberg in mixed cultures. Pol. J. Environ. Stud. 21 (5), 2012, 1399-1403.
20. Alakomi H, Skytta E, Saarela M, Mattila-Sandholm T, Latva-kalak Helander I. Lactic acid permeabilizes Grem-negative bacteria by disrupting the outer membrane. Appl. Environ. Microbiol. 66, 2000.