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Araştırma / Research Article

Laktik ve asetik asit bakterilerinin Shigella spp. Kültüründe antagonistik etkisi

Antagonistic effects of lactic and acetic acid bacteria on Shigella sp. SS10 in co-culture

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ÖZET

Giriş: Bakteri direncindeki yakın tarihli artış diyareleri de içeren enfeksiyon hastalıklarının antibiyotikle tedavisine alternatif arayışlarına neden olmuştur. Yaygın diyare etkenlerinden biri *Shigella* spp.'dir. Laktik asit bakterileri farklı diyare formlarından korunmaua yönelik probiyotik formüllerde kullanılmaktadır. Bu çalışma, laktik ve asetik bakterilerin (LAB) *Shigella* spp.'in kültürlerindeki antagonist etkisini incelemektedir. **Gereç Yöntem:** Ofloxacin, gentamycin, cefuroxime, ceftazidine, lincomycin, oxacillin, cloxacillin, cefotaxime, ciprofloxacin ve nitrofurantoine karşı *Shigella* spp.'in SS10 antibiyotik duyarlılığı, disk difüzyon yöntemi ile test edilmiştir. *Shigella* spp. SS10 eş zamanlı olarak iki farklı ortamda 3 laktobasil türü ile ((Lactobacillus plantarum QN01, Lactobacillus parabuchneri SM03 ve Lactobacillus fermetum SH01) ve daha önce Nijerya kaynaklı yoğurtlardan izole edilen Acetobacter pasteurianus RV04 ile birlikte inkübe edilmiştir. Bir 8 saatlik ve bir taze *Shigella* spp. SS10, bir gecelik LAB kültürüne eklenmiştir. Canlı patojen sayıları 37° C'da 0 ve 24. saat inkübasyonu takiben sayılmıştır. **Sonuçlar:** *Shigella* spp. SS10 test edilen patojenlerin %50'sine dirençliydi. LAB *Shigella* türlerinde canlı bakteri sayısının logaritmasını ortalama 4 azalttı. Asetobakter türleri iyi bir inhibisyonla mikrobiyal yük logaritmasını ortalama 4 azalttı. Nijerya yoğurdundan izole edilen Laktik ve asetik asit baktesisi *Shigella* spp. Eş kültüründe kayda değer azalmaya yol açtı. **Sonuç:** LAB suşları diyare gibi enfeksiyon hastalıklarının tedavisinde bir alternatif olarak araştırılabilir.

ABSTRACT

Introduction: Recent upsurge in bacterial resistance has led to search for alternative to antibiotics in treatment of infectious diseases including diarrhea. One of the common causative agent of diarrhea is *Shigella* sp. Lactic acid bacteria has been used in probiotic formulation for prevention of various form of diarrhea. This study investigates possible antagonistic effects of lactic and acetic bacteria on Shigella sp in co culture. **Material-methods:** Antibiotic susceptibility of *Shigella* spp. SS10 to ofloxacin, gentamycin, cefuroxime, ceftazidine, lincomycin, oxacillin, cloxacillin, cefotaxime, ciprofloxacin and nitrofurantoin was tested by disc diffusion method. *Shigella* spp. SS10 was co-incubated in two different experiment with 3 *Lactobacillus* species (*Lactobacillus plantarum* QN01, *Lactobacillus parabuchneri* SM03 and *Lactobacillus fermetum* SH01) and 1 *Acetobacter pasteurianus* RV04 which has been previously isolated from Nigeria-produced yogurts. An 8h old *Shigella* spp. SS10 was introduced into an overnight culture of LAB and a fresh *Shigella* spp. SS10 was inoculated into overnight culture of LAB. Viable counts of pathogens at 0h and after 24h co-incubation at 37°C were observed. **Results:** *Shigella* spp. SS10 was resistant to 50% of the tested pathogens. The tested LAB effected an average of 4 log reduction in viable counts of the *Shigella* strain. The *Acetobacter* strain displayed very good inhibitory activity with a 4 log reduction in microbial load. **Conclusion:** Lactic and Acetic acid bacteria isolated from Nigerian yoghurt has considerable activity against *Shigella* sp. in co culture experiment.

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INTRODUCTION

The gastrointestinal tract is subject to infections by many pathogens, which are a major cause of economic loss due to illness, suboptimal performance, and death. These infections spread by direct contact or the fecal-oral route. Enteric (gastrointestinal) and diarrheal disease is the second leading cause of death and the leading cause of malnutrition in children under five years old [1]. The main classes of agents that are responsible for diarrhea of infectious origin are enteropathogenic viruses and bacteria. Among the bacteria, *Shigella, Escherichia coli, Salmonella, Campylobacter* and *Vibrio* sp. are often recognized as the causative agents of diarrhea in children in developing countries [2]. These organisms ravage various regions of the world but commonly wreck havocs in the human gut in many developing countries where hygiene and health care systems are below par.

Shigella is a Gram-negative, facultative anaerobic, rod-shaped bacteria closely related to *Salmonella*, and is the causative agent of human shigellosis. It typically causes dysentery [3]. *Shigella* is one of the leading bacterial causes of diarrhea worldwide. Conservative estimates suggest *Shigella* causes about 90 million cases

of severe dysentery [4,5] with at least 100,000 of these resulting in death each year, mostly among children in the developing world, where they are major causes of moderate-to-severe diarrhea in children under age 5 [6]. Children under the age of 11 are at the greatest risk and as few as 10-200 bacteria are capable of causing disease [7]. Because of the low infectious dose, transmission can occur via contaminated food and water or via direct person-to-person spread. In the U.S., more the 75% of cases of Shigellosis are caused by *S. sonnei* while *S. flexneri* is the most prevalent species in developing countries.

The rich and complex environment of the intestine make many pathogens thrive including *Shigella* spp. in large number especially due to the ever-increasing resistance of bacterial pathogens to common and previously active antibiotics. The increasing resistances even to newer antibiotics pose a greater challenge to the individual health management system of many people living in the developing nations where sources of finance are very scarce. Furthermore, many cases of diarrhea go unidentified, often resulting in inappropriate treatment. Furthermore, continual use of antibiotics may add to the burden of the gastrointestinal environment by upsetting the balance of the protective microbiota..

Lactic acid bacteria (LAB) are a group of protective microbiota in the intestine that has been proven to be effective against many intestinal pathogens especially in probiotic formulation. Also, Acetobacter is a genus of acetic acid bacteria (AAB) characterized by the ability to convert ethanol to acetic acid in the presence of oxygen. Acetic acid bacteria are Gram-negative bacteria generally isolated from a variety of natural fields such as fruits, flowers and fermented foods. They are widely used for vinegar production because of their capacity to oxidize ethanol to acetic acid and to tolerate high concentrations of acetic acid [8]. Probiotics are products or substances containing living and potentially beneficial microorganisms and are aimed at delivering the microorganisms to the gut ecosystem of humans and animals, e.g. by restoring the balance of microflora in the digestive tract [9]. Strains of LAB are the most common microbes employed as probiotics. *L. acidophilus* has been reported to have effect on diarrhea caused by *Salmonella* or *Shigella*. While *L. casei* was reported to have curative effect on infections caused by *Salmonella* typhimurium and *E. coli* [10]. However, there is scarcity of approved probiotic LAB in Nigerian market but there are LAB and AAB added to yoghurt in Nigeria for their fermentative ability but little is known about the ability of these LAB and AAB to reduce the viable population of *Shigella* spp. This study was, therefore, carried out to determine the inhibition of growth of clinical isolates of *Shigella* spp. grown in co culture with LAB isolated from Nigerian yogurts.

MATERIALS AND METHODS

Microorganisms

Lactobacillus plantarum QN01, *Lactobacillus parabuchneri* SM03, *Lactobacillus fermentum* SH01 and *Acetobacter pasterianus* RV04 has been previously isolated from different commercial yogurts obtained from shopping malls in Ibadan, Nigeria and identified by sequencing their 16S rRNA gene. *Shigella* sp. SS10 has been previously collected from an hospital in South West, Nigeria and were further characterized by its cultural, microscopic and their biochemical characteristics according to standard procedures.

Antimicrobial susceptibility test for *Shigella* sp. SS10.

The susceptibility of the *Shigella* sp. SS10 to different antibiotics was tested using standard antibiotic disc. A 18-hr broth culture of *Shigella* sp. SS10 was inoculated onto Muller Hinton agar by spread plate method. Ten different standard antibiotic disks ofloxacin, gentamycin, cefuroxime, ceftazidine, lincomycin, oxacillin, cloxacillin, cefotaxime, ciprofloxacin and nitrofurantoin were placed on the agar plates using a sterile forceps. The plates were kept on the bench for 30 min to allow diffusion of the antimicrobials before incubating at 37°C for 24 hrs. The plates were examined for clear zones of inhibition around the discs. The diameter (mm) of zone of inhibition was measured and the result interpreted by the EUCAST Clinical Breakpoint Table version 5.0 [11].

Co-culture of LAB and *Shigella* sp. SS10

Two series of experiments were performed to examine the interference of LAB with the growth of *Shigella* spp. SS10 by coincubating the pathogen individually with four representative LAB strains from the yogurt isolates *Lactobacillus plantarum* QN01, *Lactobacillus parabuchneri* SM03, *Lactobacillus fermentum* SH01 and *Acetobacter pasterianus* RV04.

In the first experiment, *Lactobacillus plantarum* QN01 and *Lactobacillus fermentum* SH01 were grown in MRS broth for 24h in microaerophilic conditions while *Shigella* spp. SS10 was grown on Salmonella Shigella agar. From the 24h old culture, fresh *Shigella* spp. SS10 was grown for 8hrs and the resulting pathogen broth

culture was centrifuged at 4000 X g for 15 minutes, the supernatant was decanted and the pellets resuspended in fresh 5ml double-strength nutrient broth with a vortex mixer. 5ml of 8h old resuspended test pathogen culture was added to 5ml of the 24h LAB culture in MRS broth, to make a 10ml co-culture mixture. The bacterial counts were made at 8hrs and 24hrs of both the pathogen monoculture and the co-culture mixturet from appropriate dilutions on *Salmonella-Shigella* medium for viable counts of pathogens and MRS agar for LAB. The pathogen monoculture serves as control.

In the second experiment, a method described by Drago *et al.*, [2] was used and modified in this study. *Shigella* spp. SS10 was inoculated into 5 ml double strength nutrient broth and then added to the overnight culture of 5ml double strength MRS culture of *Lactobacillus plantarum* QN01, *Lactobacillus parabuchneri* SM03, *Lactobacillus fermentum* SH01 and *Acetobacter pasterianus* RV04 and incubated for 24h. Appropriate dilution of both monocultures and the mixed culture of the LAB and pathogens were evaluated at 0h by plating each LAB onto MRS agar and incubated microaerophilically at 37°C for 24hours. Monocultures and mixed cultures were plated on selective media for the respective organisms (*Salmonella-Shigella* agar for *Shigella spp.*), then incubated at 37°C for 24hours to evaluate the growth of LAB and pathogens.

RESULTS

The antimicrobial susceptibility patterns of *Shigella* sp. SS10 was performed and was found to be sensitive to ofloxacin, gentamicin, ciprofloxacin, cefotaxime and nitrofurantion and resistant to ceftazidime, lincomycin, oxacillin and cloxacillin.

Lactobacillus plantarum QN01, *Lactobacillus parabuchneri* SM03, *Lactobacillus fermentum* SH01 and *Acetobacter pasterianus* RV04 were examined for their antibacterial activities against *Shigella* sp. SS10. In the first experiment involving addition of LAB to *Shigella* sp. SS10 that has grown for 24h, *Lactobacillus plantarum* QN01 reduced *Shigella* sp. SS10 growth from $2.72x10^8$at 8h to $7.54x10^6$ in a 2 log reduction while *Lactobacillus fermentum* SH01 reduced *Shigella* sp. SS10 growth from $3.8x10^6$ at 8h to < $x10^4$ in a 2 log reduction (fig I).

In the second experiment involving co cultivation of 24h LAB and fresh *Shigella* sp. SS10, *Lactobacillus plantarum* QN01 showed no log reduction in pathogen growth, *Lactobacillus fermentum* SH01 reduced *Shigella* sp. SS10 growth by 3 log reduction (fig 1), while *Acetobacter pasterianus* RV04 and *Lactobacillus parabuchneri* SM03 reduced *Shigella* sp. SS10 growth by 4 log reduction respectively. (Table 1, fig 2). The lactic and acetic acid bacteria growth were not really affected by the pathogen.

Table 1. Viable counts of LAB and *Shigella* sp. SS10 after 24hours of co-incubation with selected LAB.

LAB			*Shigella* sp SS10	
	CFU/ml		CFU/ml	
Lb. plantarum QN01	$6.32x10^9$	($7.24x10^9$)	$1.5x10^8$	($1.03x10^8$)
Lb. parabuchneri SM03	$4.22x10^9$	($2.4x10^{10}$)	< 10^4	($1.03x10^8$)
Lb. fermentum SH01	$1.6x10^9$	($3.1x10^{10}$)	$2.6x10^5$	($1.03x10^8$)
Acetobacter pasteurianus RV04	$1.24x10^{10}$	($2.1x10^{10}$)	< 10^4	($1.03x10^8$)

Key: Values in parentheses show the growth of controls, i.e. pure culture, under the same condition.

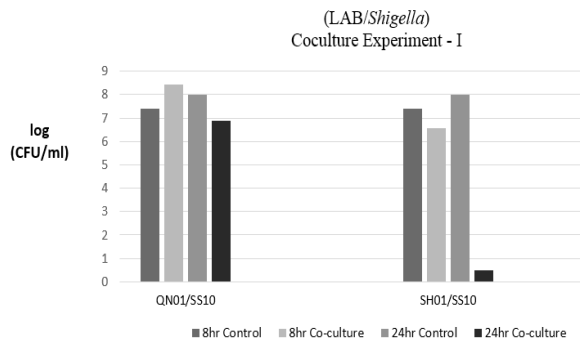

Figure 1. (Co-culture Experiment-I) Inhibition of *in vitro* growth of *Shigella* sp. SS10 by co-incubating with Selected LAB.

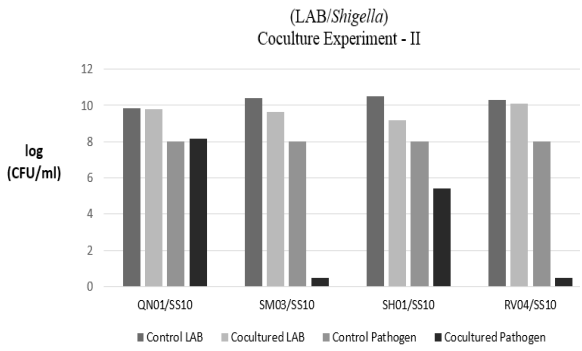

Figure 2. (Co-culture Experiment-I) Inhibition of *in vitro* growth of *Shigella* sp. SS10 by co-incubating with Selected LAB.

DISCUSSION

Shigella sp has been observed to be generally resistant to antibiotics and also one of the leading causes of enteric infections in developing countries. Antimicrobial susceptibility of *Shigella* spp. and *Escherichia coli*, isolated from diarrheal patients in Lagos, was reported by Iwalokun *et al.* [4] and found out that over 70% of the *Shigella* isolates were resistant to two or more drugs. Twenty-one distinct multidrug resistance patterns were observed in the isolates. Between 1990-2000, they reported drastic increase in resistance to drugs of choice. In this study, *Shigella* sp. SS10 was resistant to cefuroxime, ceftazidime, lincomycin, oxacillin and cloxacillin. In spite of this, ciprofloxacin and ofloxacin seemed to have been the ideal alternatives and are still active against the *Shigella* and *Escherichia coli*, but not without few resistance patterns starting to emerge at this contemporary time. Bolaji *et al.*, [12] reported the presence of antibiotic resistant bacteria in hospital waste water in Ede, Southwestern, Nigeria and that *Shigella* spp and the other organisms isolated in the study have become resistant to septrin, chloramphenicol, amoxicillin and streptomycin while they were also 90% resistant to pefloxacin, ofloxacin, 80% resistant to ciprofloxacin, 70% resistant to gentamycin. The multi-drug resistance was as a result of indiscriminate use of the antibiotics among other causes. There is in-vitro bacteriologic efficacy of gentamicin and nitrofurantoin as reported previously (4, 13) to *Shigella* spp., it still holds true for the result got in this study too, over two decades.

The LAB used in this study has a lot of antimicrobial activities on *Shigella* sp. thereby drastically reducing its log count. Four yogurt isolates which include *L. plantarum* QN01, *L. parabuchneri* SM03, *L. fermentum* SH01, and *Acetobacter pasteurianus* RV04 were used against *Shigella* sp. SS10. The *Shigella* sp. exhibited a 4-log reduction in viable count on co-incubation with the three LAB and one AAB tested after 24h co-incubation, increased acidity has been observed to have inhibitory effects on *Shigellae* [14]. The 8h co-incubation yielded less log reduction in comparism with inoculating the pathogen into fully grown LAB. There was a drastic reduction in viable counts of the pathogen after 24hrs co-incubation. *L. parabuchneri* SM03, *L. fermentum* SH01, and *Acetobacter pasteurianus* RV04 had an average of 3.67-log reduction in the viable counts of the *Shigella* sp. SS10. The result is in line with Beata *et al.*, [15], who co-cultured six *Lactobacillus* strains with *Salmonella* Senftenberg, an *Enterobacteriaceae*, and noted that all the tested LAB strains inactivated the growth of the test pathogen during a 48 h of cultivation. They team reported that the co-incubation method generally gave very good in vitro activity of the co-incubated LAB against the pathogens. *L. buchneri* have been shown to be able to resist gastrointestinal conditions and have shown potential in reducing serum cholesterol which still makes the isolate healthful [16, 17]. Amin *et al.*, [18] also isolated lactobacilli strains from fresh vegetables, and documented the remarkable antimicrobial activity exhibited by the reported lactobacilli against a panel of pathogenic bacteria such as *Escherichia coli, Salmonella typhi, Shigella dysenteriae, Bacillus anthracis* and *Staphylococcus aureus.*

In a similar fashion, AAB species, *Acetobacter pasteurianus* use in this study also displayed good antibacterial activities against the *Shigella* sp. Acetic acid has been shown to have good antibacterial activity against micro-organisms such as *Pseudomonas aeruginosa* [19]. The ability of AAB to inhibit microbial growth could not be much surprising since they have been found to produce appreciable quantity of acetic acid which may have inadvertently reduce the pH of the environment thereby suppressing the growth of the tested pathogen.

The growing concern about the presence of and the spread of multidrug resistant gastrointestinal species was emphasized by the findings earlier discussed thereby underscoring the need for rational application of antibiotics and other necessary interventions that will help to control the menace of antibiotic resistance [20]. The multiresistant *Shigella* sp. examined in this study have higher susceptibility to LAB and AAB relative to the antibiotics used against them. Therefore, the yoghurt from Nigeria have LAB and AAB in them with strong inhibitory effects against *Shigella* sp. in co culture experiment.

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