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In vitro Studies on Antimicrobial Activities of Lactic Acid Bacteria Isolated from Fresh Vegetables for Biocontrol of Tomato Pathogens

E. C. Emerenini[1], O. R. Afolabi[2], P. I. Okolie[3*] and A. K. Akintokun[2]

[1]Department of Agricultural Biotechnology and Bio-Resources, National Biotechnology Development Agency, (NABDA), Abuja, Nigeria.
[2]Microbiology Department, Federal University of Agriculture, PMB 2240, Abeokuta, Ogun State, Nigeria.
[3]Biotechnology Centre, Federal University of Agriculture, PMB 2240, Abeokuta, Ogun State, Nigeria.

Authors' contributions

This work was carried out in collaboration between all authors. Authors ORA and AKA supervised the study. Author ECE designed and wrote the protocol. Authors ECE and PIO wrote the first draft of the manuscript and managed manuscript corrections. Authors ECE and PIO managed the analyses of the study. Author ECE managed the literature searches. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: This study was focused on using Lactic Acid Bacteria (LAB) isolated from fresh vegetables which has been molecularly identified for in vitro control of some tomato pathogens.
Study Design: The inhibitory potentials of supernatant obtained from previously characterized LAB isolates or vegetable origin were investigated against some tomato phytopathogens using agar-well method with the view to develop biological agents for some tomato disease causing organisms.
Place and Duration of Study: Biotechnology Centre of Federal University of Agriculture, Abeokuta, Ogun State, Nigeria, between January 2011 and February 2012.
Methodology: The antimicrobial activities of LAB against some tomato phytopathogenic bacteria which include (*Xanthomonas campestries, Erwinia caratovora, and Pseudomonas syringae*) were obtained by using the agar well diffusion method.
Results: The result indicates that cell free culture of LAB from fresh vegetables origin

**Corresponding author: Email: piusokolie@yahoo.com; okoliepi@Funaab.edu.ng;*

(*Weissella paramesenteroides, Lactobacillus pentosus, Weissella cibaria, Pediococcus pentosaceus, Weissella kimchi* and *Lactobacillus plantarum)* can inhibits these bacteria by creating clear zones of inhibition around the wells containing cell free supernatants of the above mentioned strains of lactic acid bacteria. *Pediococcus pentosaceus* showed the highest zone of inhibition against *Xanthomonas campestries at* 15 mm radius*, Weissella kimchi* was the least effective against *Pseudomonas syringae* at 3.67 mm and *Erwinia caratovora* at 3.50 mm radius.

Conclusion: Tomato disease causing organisms can be most likely biologically controlled by using extracts from LAB. This finding will reduce the potential hazard from the use of chemical herbicides on plant.

Keywords: Lactic Acid Bacteria; Antimicrobial activities; Pathogens; Bacterioci; Inihibition.

1. INTRODUCTION

Tomato (*Lycopercicum exculentum*) is one of the most important vegetables in many parts of the word. It is a vegetable crop of considerable economic importance worldwide. It is very important in the diet of Nigerians and serves as a cash crop for many farmers too [1]. The record have shown that Nigeria produces approximately 1.8 million metric tons of fresh tomatoes for domestic consumption, with national demand of about 2-3 million metric tons annually. This marginal gap of about 500 million metric tons needed to meet up with demand index is costing the Federal Government over the N11.7 billion annually on the importation of processed tomato paste. One of the causal factors in the poor production of tomatoes in the country is associated with pre and post-harvest diseases caused by plant phytopathogens. Fruits of different cultivars of tomato vary greatly in their susceptibility to disease [2]. Physiological stress on the fruit, mechanical damage and injuries during and after harvest creates infection root for pathogens [3]. Some microorganisms has been implicated as a major cause of tomato fruit and other fruit diseases in many countries and has been described as common spoilage microorganisms of fresh fruits and vegetables [4]. Such organisms include *Erwinia carotovora Xanthomonas vesicatoria* and *Pseudomonas syringae* [3,4].

Many LAB strains are able to produce protein compounds with efficient antimicrobial effect, which are known as bacteriocins [5]. The bacteriocin from LAB can be categorized into three distinct class: (i) lantibiotics or small, heat-stable, lanthionine-containing, single-and two-peptide bacteriocins (class I), whose inactive prepeptides are subject to extensive post translational modification; (ii) peptide bacteriocins or small, heat-stable, non-lanthionine-containing bacteriocins (class II), including pediocin-like or *Listeria*- active bacteriocins (class IIa), two-peptide bacteriocins (class IIb), and circular bacteriocins (class IIc), and, arguably, (iii) bacteriolysins or large, heat-labile, lytic proteins, often murein hydrolases (class III) [6]. The inhibition of pathogenic growth occurs because LAB is highly effective in microbial antagonism. These LAB have the ability to outcompete other microorganisms for nutrients and residency. These following strains of *Enterococcus faecium, Streptococcus thermophilus, Lactobacillus casei and Lactobacillus sakei* subsp. *sakei* were characterizied using 16S rRNA gene sequencing and has been identified as bacteriocin-producing lactic acid bacteria against *L. innocua, Escherichia coli, Bacillus cereus, Pseudomonas fluorescens, Erwinia carotovora, Penicillium expansum, Botrytis cinerea and Monilinia frucitcola* [7]. There is a complimentary effect by the production of acid and antimicrobial compounds that increases inhibition of both pathogen and spoilage bacteria [8].

Although many efforts have been made to develop bioprotective lactic acid bacteria strains, the application of these strains in fresh fruits and vegetables have not been developed yet [9].

The aim of this work was to study the in-vitro effect of the cell free culture of already characterized LAB isolated from fresh vegetables as an antimicrobial compound to inhibit the growth of some gram negative pathogenic bacteria associated with tomato spoilage.

2. MATERIALS AND METHODS

2.1 Isolation of LAB

Fresh vegetables, flutted pumpkin vegetable (*Telfairia occidentalis*) and green vegetable (*Amaranthus spinosus)* were obtained randomly from local markets in Abeokuta town, Ogun State, Western part of Nigeria. Ten grams of each sample were homogenized for 2 min in 90ml of sterile normal saline solution (NaCl, 8.5 g/L). Serial dilutions up to 10^{-5} were prepared and appropriate dilutions were plated by pour plate method onto de Man Rogosa and Sharpe agar (Lab M). Duplicate plates were incubated anaerobically at 37ºC for 48-72 h, distinct colonies were sub-cultured and pure cultures were stored in McCartney bottles for further study.

2.2 Test Organisms Used in this study

The test isolates (*Erwinia carotovora. Xanthomonas vesicatoria* and *Pseudomonas syringae)* were collected from the Department of Microbiology, Federal University of Agriculture Abeokuta, Nigeria.

2.3 Preparation of LAB Cell-Free Filtrate

MRS broth (1000 µl) were inoculated separately with LAB previously characterized [10] and incubated at 37ºC for 72hrs. After incubation, a cell free supernatant was obtained by centrifuging (Spectrafuge 24D, Labnet, USA) the bacterial culture at 10,000 rpm for 45 min, followed by filtration of the supernatant through 0.2 mm pore size filter paper thus obtaining cell free filtrate [11].

2.4 Preparation of Cell Culture from Phytopathogens

One thousand microliters of Tryptic Soy Broth (TSB) was inoculated separately with each of the phytopathogens and incubated at 28ºC overnight. After incubation, the culture of each isolate was standardized to an optical density of 0.5 at a wavelength of 600 nm (Hitachi U-2010 model Spectrophotometer). The cell culture obtained was used for the detection of antimicrobial activity of LAB by agar well diffusion**.**

2.5 Screening of LAB for Antimicrobial Activity

Screening of lactic acid bacteria for antimicrobial activity was carried out according to the method described by [12], with some modifications.

The modifications introduced are as follows:

a. The centrifugation was at 10,000 rpm for 45 min,
b. The LAB cells were incubated in MRS broth at 37ºC for a longer time (72hrs).

After incubation at 37ºC for 72 hrs, cells were removed by centrifugation at 10,000 x g for 15 min. The filtrates were used to evaluate antimicrobial activity using agar well diffusion method. Positive results were recorded when the zone of inhibition of at least 1 mm around the wells was observed.

2.6 Detection of Antimicrobial Activity by Agar Well Diffusion Method

An agar well diffusion method was used [13]. Petri dishes containing 20 ml of TSA were previously prepared and a lawn of each indicator strain was made by spreading 100 µl of 24 hrs standardized broth culture of pathogenic bacteria over the surface of TSA plates, with sterile glass spreader. The plates were allowed to dry and a sterile cork borer of diameter 5.0 mm was used to make uniform wells in the agar plates. Each well was filled with 100µl of filter sterilized supernatant obtained from LAB cultures grown in MRS broth and incubated at 30ºC for 24 hrs. The inhibitory activity was determined by measuring the radius of inhibition zone around the well with the aid of a pair of divider and meter rule in mm [11]. All the assays were carried out in triplicate.

2.7 Statistical Analysis of Zones of inhibition of CFS against Phytopathogens

The data from the zones of inhibition created on lawns of phytopathogens by LAB CFS were determined by One-way ANOVA analysis using SPSS statistical software version 13. The standard mean deviations of the zones of inhibition were also determined for each mean value. The mean were separated using Duncan test with p-value of $P \leq 0.05$.

3. RESULTS AND DISCUSSION

Lactic acid bacteria were isolated from fresh vegetables on MRS medium. Antimicrobial activities of the isolated LAB were tested against the tomato phytopathogens. Table 1 show the molecularly characterized organisms used in this study.

The percentage abundance of LAB organisms identified from fresh vegetable was summarized in Table 2.

Table 3 shows the statistical analysis of antimicrobial activity of CFS of lab from vegetables against Phytopathogens.

Fig. 1 show inhibition pattern of CFS of some LAB against tomato pathogenic bacteria used in this study.

Table 1. Lactic Acid Bacteria from fresh vegetable used for this study.

Isolate code of organisms identified	Reference from NCBI database	Nucleotide number	Percentage similarity (%)	NCBI Accession number
AU2	*Weissella paramesenteroides*	1414	99	FJ405229.1
AU3	*Weissella cibaria*	1484	100	AB362617.1
AU4	*Lactobacillus plantarum*	1471	100	GU552552.1
AU5	*Lactobacillus plantarum*	1472	99	FJ386491.1
AU7	*Weissella paramesenteroides*	1431	99	AB362621.1
BU2	*Pediococcus pentosaceus*	1481	100	AB481102.1
BU3	*Weissella cibaria*	1488	100	AB362617.1
BU8	*Weissella paramesenteroides*	1480	99	FJ405229.1
CU2	*Lactobacillus plantarum*	1363	100	GU552552.1
AA2	*Weissella cibaria*	1482	100	AB362617.1
AA3	*Lactobacillus plantarum*	1475	100	FJ386491.1
AA8	*Weissella cibaria*	1484	99	AB362614.1
AA10	*Weissella kimchi*	1484	99	AF312574.1
BA3	*Lactobacillus plantarum*	1471	100	DQ239698.1
BA4	*Weissella kimchi*	1480	99	AF312874.1
BA7	*Pediococcus pentosaceus*	1481	100	FM179610.1
BA8	*Weissella cibaria*	1474	99	GU138579.1
CA6	*Lactobacillus pentosus*	1477	100	AB362758.1

*These LAB Isolates were previously characterized using molecular method [10]

Table 2. The percentage abundance of organisms identified from fresh vegetables.

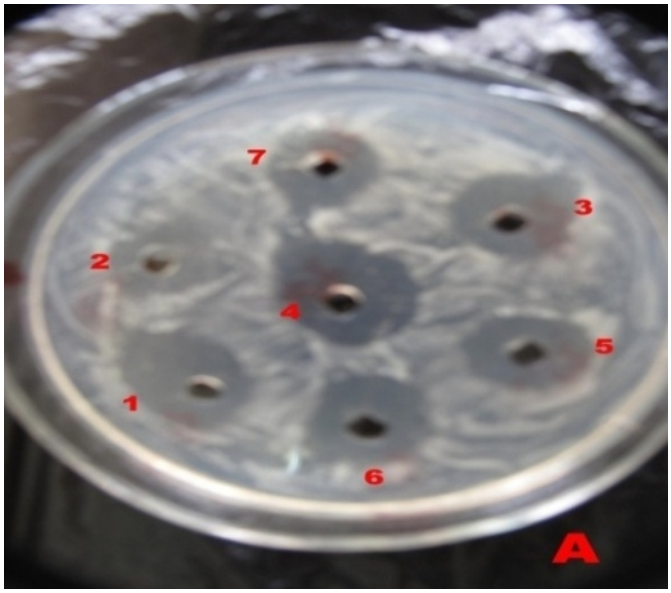
Organisms identity	Percentage abundance (%)
Weissella cibaria	5 (27.78)
Weissella kimchi	2 (11.11)
Weissella paramesenteroides	3 (16.67)
Lactobacillus plantarum	5 (27.78)
Pediococcus pentosaceus	2 (11.11)
Lactobacillus pentosus	1 (5.56)

As the result indicates, The cell free supernatant of LAB strains exhibited antimicrobial potentials and gave the radius zone of inhibition onto indicator pathogenic strains ranging between 4mm and 15mm. Inhibition was graded positive if clear zone around the well loaded with CFS of LAB was 1mm and above [14]. Pediococcus pentosaceus showed the highest zone of inhibition against Xanthomonas campestries at 15 mm radius and thus are very sensitive to the CFS from Pediococcus pentosaceus, CFS from Weissella cibaria is relatively sensitive against Erwinia caratovora with zone of inhibition of 10 mm radius, and CFS from Weissella kimchi and W. cibaria is shown to be sensitive against Pseudomonas syringae at inhibition zone of 13 mm while Lactobacillus pentosus showed to be least sensitive on Xanthomonas campestries at inhibition zone of 5 mm, Weissella kimchi was the least sensitive against Erwinia caratovora at inhibition zone of 3.50 mm, and Pseudomonas syringae at inhibition zone of 3.67 mm as represented in Table 3.

Table 3. Statistical analysis of antimicrobial activity of CFS of LAB isolates from fresh vegetables against tomato phytopathogens

LAB Isolate	*Xanthomonas campestries* (measured in mm)	*Erwinia caratovora* (measured in mm)	*Pseudomonas syringae* (measured in mm)
Weissella paramesenteroides	12.00 ± 1.00^{aAB}	5.00 ± 2.00^{bCDE}	10.00 ± 2.00^{aABC}
Weissella cibaria	Nil	8.00 ± 1.73^{bABC}	13.00 ± 3.00^{aA}
Lactobacillus plantarum	Nil	8.00 ± 2.65^{aABC}	9.00 ± 1.00^{aABCD}
Lactobacillus plantarum	10.00 ± 2.65^{aB}	5.00 ± 1.00^{bCDE}	7.00 ± 2.00^{abBCDE}
Weissella paramesenteroides	12.00 ± 2.65^{aAB}	5.17 ± 1.26^{bCDE}	Nil
Pediococcus pentosaceus	6.00 ± 3.46^{bCD}	5.00 ± 1.00^{bCDE}	5.00 ± 1.73^{bDE}
Weissella cibaria	Nil	10.00 ± 3.00^{aAB}	11.00 ± 1.00^{aAB}
Weissella paramesenteroides	9.00 ± 1.00^{abBC}	10.33 ± 2.08^{aA}	4.00 ± 1.73^{cEF}
Lactobacillus plantarum	11.00 ± 2.65^{aB}	Nil	Nil
Weissella cibaria	6.00 ± 1.00^{bCD}	6.50 ± 1.32^{bCDE}	12.00 ± 4.36^{aA}
Lactobacillus plantarum	Nil	5.00 ± 1.00^{bCDE}	9.00 ± 2.00^{aABCD}
Weissella cibaria	11.00 ± 2.65^{aB}	8.00 ± 1.00^{abABC}	6.00 ± 2.65^{cCDE}
Weissella kimchi	6.00 ± 2.00^{bcCD}	3.50 ± 0.87^{cE}	13.00 ± 2.65^{aA}
Lactobacillus plantarum	5.00 ± 2.00^{bD}	4.33 ± 1.53^{bDE}	10.00 ± 2.65^{aABC}
Weissella kimchi	5.00 ± 1.00^{bD}	Nil	3.67 ± 1.15^{bEF}
Pediococcus pentosaceus	15.00 ± 2.00^{aA}	7.00 ± 2.00^{bBCD}	7.00 ± 2.65^{bBCDE}
Weissella cibaria	6.00 ± 2.65^{bCD}	10.00 ± 2.65^{aAB}	Nil
Lactobacillus pentosus	Nil	5.00 ± 2.65^{bCDE}	12.00 ± 3.61^{aA}

Data were presented as mean (measured in mm) ± standard deviation
Means with the same small letter across the row are not significantly different at P ≤ 0.05.
Means with the same capital letter across the column are not significantly different at P ≤ 0.05.



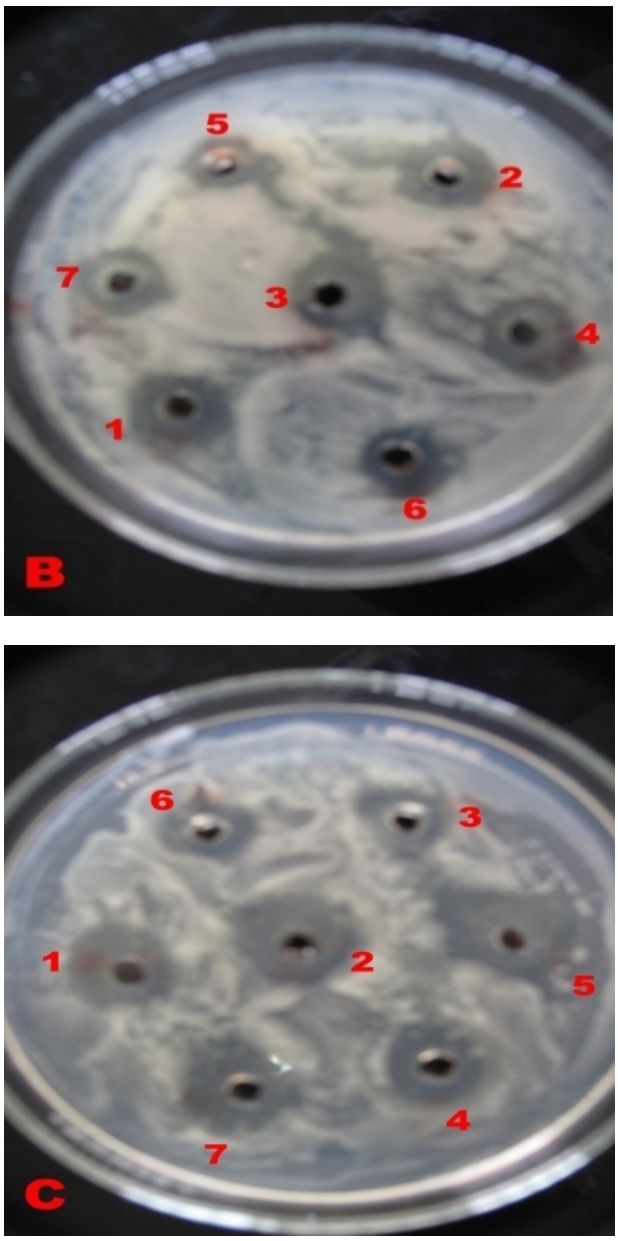


Fig. 1. Inhibition zones of CFS of some LAB strains against tomato pathogenic bacteria. 1- *Weissella paramesenteroides,* 2- *Lactobacillus pentosus,* 3- *Weissella cibaria,* 4- *Pediococcus pentosaceus,* 5- *Weissella kimchi,* 6- *Lactobacillus plantarum,* 7- *Lactobacilus plantarum.*
A= *Xanthomonas campestries*, B= *Erwinia carotovora* and C= *Pseudomonas syringae*

This is an indication that the strains of LAB used inhibited all the pathogenic bacteria tested in this study. This research relates to the study on using the extracts from LAB against some pathogenic *E. coli, Enterococcus faecalis, Staphylococcus aureus and Bacillus cereus* [15].

The findings of this study are in conformity with the work on grading positive inhibition when the width of clear zone around the well was 0.6mm or larger [16]. Their result revealed inhibition zone which ranged between 0.6 and 4mm.

Lactic acid bacteria synthesize bactericidal agents that vary in their spectrum of activity [17]. Such compounds consist of hydrogen peroxide, organic acids, lytic agents, bacteriocins or antimicrobial peptides, diacetyl, defective phages and enzymes [18]. Researchers has observed that the mechanism of action for LAB antimicrobials is the disruption of the cytoplasmic membrane of susceptible bacteria by forming apertures in their membrane resulting in increased permeability to small compounds [8].

Therefore, cell free culture of LAB isolated from vegetables can act as the best agent for microbiological safety of tomato and perhaps other fruits and act as a barricade to microbial spoilage and or growth of pathogenic bacteria.

4. CONCLUSION

In view of the fact that LAB is generally regarded as safe (GRAS), the antimicrobial substances produced could control the major postharvest diseases of tomato and other fruits and food products in Nigeria. This study has shown that lactic acid bacteria isolated from fresh vegetables, have potential to serve as biocontrol agents for tomato pathogens in-vitro. They can act as the best substitutes for improving the microbiological safety in biopreservation of tomato fruits. This research also has provided the basis for in-vivo application of the findings for the elimination of the tomatoes diseases causing bacteria.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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