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IDENTIFICATION, ANTAGONISTIC ACTIVITIES AGAINST *VIBRIO CHOLERAE* 01 OF LACTIC ACID BACTERIA ISOLATED FROM NIGERIAN GROWN SALAD VEGETABLES

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Abstract: Lactic acid bacteria (LAB) are isolated from different niches and have been reported to exhibit inhibitory activities against pathogens of humans, animals and plants. This study which to the best of our knowledge, is the first in Nigeria, investigated the identities using partial sequencing of 16SrRNA genes and anti-Vibrio activities of LAB isolated from Nigerian grown salad vegetables. The vegetables, cabbage, carrot, cucumber and lettuce procured from 12 sales spots in Lagos Nigeria were processed using standard Microbiological procedures. Each vegetable was inoculated into sterile de Man Rogosa Sharpe (MRS) broth, incubated in 5% CO_2 atmosphere for initial 18hrs at 37^{o}C. Subculture for distinct colonies was made from this into sterile MRS agar plate and incubated further for 24 hrs. Amplification, using Polymerase chain reaction (PCR) and partial sequencing of 16S rRNA of all Gram positive, catalase, oxidase negative, non spore forming colonies were carried out. The cell free supernatants (CFS) of randomly selected eight species of the confirmed LAB were employed in the antagonistic assays against four outbreak strains of *Vibrio cholerae* 01 in an agar well diffusion assay. A total of 96 LAB isolates showing amplification of 16S rRNA gene were sequenced. The sequences showed between 89-99% similarities to 16 different LAB species in GeneBank as follows; *Lactobacillus* spp (8), *Weissella* spp (3), *Pediococcus* spp (3), Uncultured solibacillus (1), and *Enterococcus durans* (1). These are categorized into 37 different strains. The CFS of LAB showed varied zones of inhibition against *V. cholerae* 01. The highest range was between 14- 22 mm from *W. confusa* while the lowest, 8- 12mm was from *P. pentosaceus*. These were isolated from cabbage and cucumber respectively.

Keywords: Salad vegetables, LAB, 16S rRNA, Sequencing, *V. cholerae,* Antagonistic.

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INTRODUCTION

Fresh produce like fruits and vegetables are a part of human diets in different climes and are consumed in large quantities. Vegetables are traditionally regarded as microbiologically safer than other unprocessed food items such as meat, milk, eggs, sea foods et cetera. They produce a suitable abode for bacteria, moulds and yeast (Mahrosh et al. 2012; Trias et al. 2008; Wiessinger et al. 2000)

Lactic acid bacteria (LAB) have been reportedly isolated from grains, dairy and meat products, vegetables and mucosal surfaces of animals (Lindgren & Dobrogosz 1990; Patil et al. 2007). The isolation of LAB from cucumber and carrot was differently reported (Atul & Ramesh 2008; Kingston et al. 2010; Patil et al. 2010). Some of these exhibited antimicrobial properties against a wide range of plants and animal pathogens, even the multi drug resistant ones.

Vibrio cholerae is the causative agent of cholera; a harsh, diarroeal disease occasioned by rice water stooling, dehydration, acidosis and eventually death if proper treatment is not provided. Some attempts have been made to challenge this bacterium using either the metabolites or co-culture with LAB. For instance, Mahbubul et al. 2012; Vidya et al. 2010 demonstrated anti-vibrio activities of LAB isolated from carrot while Jari et al. 2012 reported the activities of *Lactobacillus rhamnosus* GG and *Bifidobacterium longum* 46 against cholera toxin.

In Nigeria, although LAB have been shown to exhibit some antimicrobial properties (Adeniyi & Iveren 2011; Adeniyi et al. 2006; Bamidele et al. 2013), there is paucity of information on their anti- vibrio activities in spite of the fact that cholera disease has become endemic in the country with the emergence of some multi drug resistant strains.

There is no information on the genotypic identification of LAB resident on salad vegetables grown in Nigeria to the best of our knowledge hence, this study was designed to investigate the identities employing molecular tools such as sequencing of 16SrRNA gene and activities against *Vibrio cholerae* 01 of these bacteria.

MATERIALS & METHODS

The vegetables investigated were Cabbage, Carrot, Cucumber and Lettuce. They were procured from 12 vegetable sales spots in Lagos state, Nigeria and transported to the laboratory immediately for processing.

Processing: The vegetables were blended following the standard procedures; briefly each vegetable (5g) was loaded into sterile blender (Q- link, Japan), transferred into 45ml of sterile de Man Rogosa Sharpe (MRS) broth in falcon tubes and incubated for initial 18 hrs at 37^0C in the atmosphere of 5% CO_2 (Microaerophilic). Inoculum was taken from this into sterile MRS

agar plates and incubated further for 24 hrs under same conditions as above. The plate was read after this period.

Isolation of LAB: The 24hr culture was subcultured into fresh sterile plate to get distinct colonies. All the different distinct Gram positive, non spore forming, catalase, oxidase negative colonies were frozen (-80^{o}C) in 20% glycerol for further assays.

DNA Extraction: This was done using Zymo Research DNA Miniprep (Zymo Research, USA) according to the manufacturers' protocol. The purity/ concentration of the extract was confirmed with Nano drop spectrophotometric instrument.

Amplification of 16SrRNA gene: The set of primers; BSF- 8(5^{1}- AGAGTTTGATCCTGGCTCAG- 3^{1}) and BSR- 534 (5^{1}- ATTACCGCGGCTGCTGGC- 3^{1}) were employed (Wilmotte et al. 1993) and 5X HOT Firepol mastermix (Solis biodyne, Estonia) used in a PCR tube in which 2µl of template DNA has been added.

The reaction mixture was done following the manufacturers' protocol. This mixture, carried out in an (vapo.protect) Eppendoff Mastercycler, was incubated at 95^{o}C for 15 mins followed by 40 cycles at 94^{o}C for 30s, 55^{o}C for 30s, and 72^{o}C for 30s.

Agarose gel electrophoresis:

The PCR product (5µl) was loaded alongside a 100bp marker (Promega, USA) on a 1% agarose stained with 0.01% ethidium bromide and electrophesis run for 1hr. The gel was viewed under ultraviolet (UV) ray in gel photodocumentation system (Clinix, China).

All the amplicons showing bands corresponding to 526 bp were sent to GATC, Germany for commercial 16S rRNA partial sequencing. The sequences generated were aligned with the ones in GeneBank (NCBI) using basic local alignment search tool (BLAST).

LAB vs *Vibrio cholerae* 01:

The *Vibrio cholerae* for this assay were outbreak strains from Nigeria. They were confirmed, using combinations of cultural, Biochemical and PCR assays.

The confirmed LAB were grown in MRS broth according to the conditions above. The broth cultures were centrifuged at 10,000g for 10 mins and the supernatants filtered using 0.22µm Millipore filter.

The resultant filterates (cell free supernatants, CFS) were used in agar well diffusion antagonistic assay as follows;

One hundred (100µl) microliter of CFS was transferred into a well bored, using a sterile cork borer (6mm diameter) in a Mueller Hinton agar (MHA) plates previously seeded with normal saline suspended *V. cholerae* equivalent to 0.5 Mac Farland standard (10^8 CFU/ ml). The plates were incubated in air, at 37^{o}C for 24 hrs and the zones of inhibition (in millimeter) measured. The control, comprising of the sterile, uninoculated MRS broth was incubated in same conditions as test.

RESULTS

A total of 96 LAB isolates that showed amplification of 16SrRNA gene were sequenced. The sequences showed between 89- 99% alignment to 16 different LAB species that are categorized into 37 strains (Table 1).

Twenty of the LAB representing 22% of total, had excellent alignments with *Weissella confusa* and this was more common in cabbage (8), followed closely by lettuce (7). *Lactobacillus plantarum* was the next common in all the vegetables.

Table 1: The Strains of LAB isolated from salad vegetables.

LAB	Strain (n)
Weissella cibaria	PON10339 (9)
	RBA-12 (3)
	qz-70 (1)
Pediococcus pentosaceus	Wg8 (1)
	FMAC63 (5)
	LAB6 (5)
	FMAC63 (1)
Pediococcus acidilactici	Mashack1 (1)
	LAB5 (2)
	FMAC22 (1)
	MW2 (3)
Uncultured *solibacillus*	Clone RBL-135 (1)
Lactobacillus plantarum	KC31 (1)
	YML007 (1)
	TCP008 (6)
	R30-1 (1)
	RU24-1 (5)
	Fish-45 (1)
Lactobacillus fermentum	LG1 (1)

	PON45 (8)
	Chilika Lbc-5 (1)
	PBCC11 (1)
	MRTL8 (1)
Weissella confusa	FS027 (15)
	PD416 (3)
	SJL602 (1)
	qz-484 (1)
Lactobacillus sp	THK- V8 (1)
Lactobacillus brevis	BB7 (4)
Pediococcus dextrinicus	Clone P17 (1)
Lactobacillus vaginalis	FQ097 (1)
Lactobacillus johnsonii	MH8 (1)
	JCM2012 (1)
Lactobacillus reuteri	KLDS 1.0736 (1)
Lactobacillus paralimentarius	IMAU:10281 (1)
Weissella paramesenteroides	69DCEP01MX (1)
Enterococcus durans	R02-22 (1)

There are some LAB that were common to a particular vegetable, for instance, *Weissella paramesenteroides* was isolated from cucumber, *Lactobacillus vaginalis* and *Enterococcus durans* were from lettuce, *Lactobacillus reuteri* and *Lactobacillus paralimentarius* from carrot only (Table 2).

The cell free supernatants (CFS) of LAB against the outbreak strains of *V. cholerae* showed varied zones of inhibition with the highest range seen against *V. cholerae* 4.

Although *W. confusa* isolated from cabbage showed the highest range of inhibition zone (14-22mm), the CFS of this isolate from cucumber showed lowest range of zone against the study pathogen which was between 8- 12 mm diameter. The CFS of *P. pentosaceus* isolated from cucumber showed similar range against *V. cholerae* 2 (Table 3). The controls did not show any zones of inhibition.

Table 2: The isolated LAB spp from different salad vegetables.

LAB	Cabbage (n)	Carrot (n)	Cucumber (n)	Lettuce (n)	Total (n)
W. confusa	8	2	3	7	20
W. cibaria	4	5	2	2	13
W. paramesenteroides	-	-	1	-	1
***Lactobacillus* spp**	1	-	-	-	1
L. fermentun	4	1	5	2	12
L. plantarum	7	2	1	5	15
L. reuteri	-	1	-	-	1
L. paralimentarius	-	1	-	-	1
L. brevis	-	2	1	-	3
L. johnsonii	-	-	1	1	2
L. vaginalis	-	-	-	1	1
P. pentosaceus	2	1	6	3	12
P. dextrinicus	1	-	-	-	1
P. acidilactici	-	-	5	2	7
Uncultured solibacillus	-	-	1	-	1
E. durans	-	-	-	1	1

Table 3: The inhibitory zones of LAB against *V. cholerae* 01

		Inhibition zones diameter range (mm)			
LAB	SOURCE	*V. cholerae* 1	*V. cholerae* 2	*V. cholerae* 3	*V. cholerae* 4
P. pentosaceus	Cucumber	10- 15	8- 12	9- 12	13- 15
	Cabbage	12- 14	12- 14	12- 14	14- 16
P. acidilactici	Cucumber	11- 13	11- 12	14- 15	14- 15
	Lettuce	11- 13	9- 12	10- 14	13- 18
W. confusa	Cucumber	10- 15	8- 12	10- 12	14- 15
	Cabbage	10- 14	9- 12	10- 16	14- 22
	Lettuce	-	10- 12	10- 15	10- 15
L. plantarum	Cucumber	-	-	-	14
	Cabbage	-	-	-	15
L. fermentum	Cucumber	-	10- 12	12- 16	14- 16
	Lettuce	10- 14	10- 13	12- 13	12- 20
W. cibaria	Cucumber	-	-	-	19
	Cabbage	-	-	15	12- 15
***Lactobacillus* spp**	Cucumber	-	-	11	-
P. dextrinicus	Cabbage	-	-	13	14

No inhibition

DISCUSSION

In Nigeria and indeed world over, salad vegetables such as cabbage, carrot, cucumber and lettuce are served and eaten raw as supplements to foods like grains in parties, homes and social functions. These vegetables are grown in some parts of Nigeria using animal manure and even human faeces. Previously, the LAB residents in these niches indicated *L. cellobiosus* and *L. salivarius* as parts of the flora (Bamidele et al. 2013). The present study did not isolate these 2 LAB even though carrot was included this time around. This may be attributed to the fact that API 50CH sugar fermentation system applied solely as a means of identification during that study was not discriminatory enough compared to partial sequencing of 16S rRNA which was employed in this study. The application of this molecular tool has allowed a wider identification of LAB resident in the salad vegetables. To the best of our knowledge, this is the first time the LAB resident in Nigerian grown salad vegetables will be speciated using molecular tool like 16S rRNA sequencing. Kingston et al. (2010), using combinations of repetitive PCR (rep- PCR) and 16SrDNA sequencing identified *L. plantarum* to be associated with carrot fermentation. In our study, *L. plantarum* was isolated from the four vegetables. It is interesting to note that 2 of our isolates had excellent alignments of 99% and 95% respectively with cloned *P. dextrinicus* and uncultured Solibacillus (Table 1). These were isolated from cabbage and cucumber respectively.

CFS of eight species of the LAB were used to challenge *V. cholerae* 01 in agar well diffusion assay. Their antagonistic activities were not unexpected as the pathogen is known to have predilection for basic pH (8.5). The pH range of the LAB isolated from the study vegetables is between 4.06- 4.9 (Data unpublished). Four outbreak strains of *V. cholerae* 01 were tested in this study and these were isolated from four different spots in Nigeria.

Studies have reported LAB anti- vibrio activities which are either against the virulence factor or the cholera bacterial pathogen itself.

For instance, Jari et al. (2012) reported the in- vitro removal of cholera enterotoxin by *Lactobacillus rhamnosus* GG and *Bifidobacterium longum* 46, while others either reported direct contact of the LAB with the pathogen or the supernatants exhibiting inhibitory activities (Gibson & Wang 1994; Mahbubul et al. 2012; Senthilkumar et al. 2012; Simonetta et al. 1997; Vidya & Iyer 2010). LAB generally seem to exhibit more antagonism when in cell- cell contact with other bacteria. This has been variously reported (Savino et al. 2011; Schellenberg et al. 2006). Thus we would like to see a trial where probiotic LAB are packaged to be taken orally by cholera patients.

The result in this study indicated organic acids to be responsible for the inhibitory activities observed, as the neutralized CFS of the LAB did not show any inhibition although this is not to out rightly infer that none of the isolates could produce other metabolites such as bacteriocins,

hydrogen peroxide et cetera, which are antimicrobials. The culture system has to be adjusted in most cases, to favour the production of these metabolites especially bacteriocins.

Although more discriminatory molecular tools such as rep- PCR, Multi locus sequence typing (MLST) would have been of greater values in identifying the LAB residents in the study vegetables, 16SrRNA partial sequencing employed in this study is a good start, given the paucity of information on molecular identification of LAB species in these vegetables in Nigeria. These bacteria have been found by this study to exhibit antagonistic activities against outbreak strains of *V. cholerae* 01, the implication of which is the possibility of exploiting some of the LAB as probiotics against this pathogen.

CONCLUSION

Although more discriminatory molecular tools such as rep- PCR, Multi locus sequence typing (MLST) would have been of greater values in identifying the LAB residents in the study vegetables, 16SrRNA partial sequencing employed in this study is a good start, given the paucity of information on molecular identification of LAB species in these vegetables in Nigeria. These bacteria have been found by this study to exhibit antagonistic activity against outbreak strains of *V. cholerae* 01, the implication of which is the possibility of exploiting some of the LAB as probiotics against this pathogen.

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