Biocontrol Activity Of Lactic Acid Bacteria Metabolites Against Bacteria Associated With Contamination And Spoilage Of Fruits And Vegetables

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Abstract
Lactic acid bacteria (LAB) strains were isolated from fermented cassava tubers and the activities of the resultant secondary metabolites assessed as biocontrol agents against two bacterial pathogens *(Escherichia coli* O157:H7 and *Erwinia carotovora*) associated with contamination and spoilage of fresh fruits and vegetables. Twenty (20) LAB isolates were identified according to standard morphological and biochemical methods and ten were further subjected to phenotypic and genotypic identification. The LAB isolates were identified as *Lactobacillus pentosus* strains PIS23 and Reyan20, *Lactobacillus plantarum* strains PON10014, CTBRBL268 and N3114, *Lactobacillus brevis* strain NS25, *Lactobacillus delbrueckii* strain NS9, *Lactobacillus fermentum* strain NS9, *Lactococcus lactis* strain NS32 and *Leuconostoc mesenteroides* strain NS73. The metabolites were tested for their anti-bacterial activity using agar-well diffusion method. The LAB metabolites were ascertained to contain bacteriocin, lactic acid, diacetyl and hydrogen peroxide, and showed strong inhibitory activity against the target bacteria. The largest zone of inhibition was 23mm (23 ± 0.289^{d}) produced by metabolites of *Lactobacillus plantarum* against *Erwinia carotovora*. Preliminary study of applying the LAB metabolites on fresh fruits and vegetables for inhibition of the growth of the target bacteria was performed using the metabolites as sanitizer and biocontrol agent. This study demonstrates that metabolites from lactic acid bacteria have the potential to be used as biocontrol agent against various bacterial pathogens associated with spoilage and contamination of fresh fruits and vegetables as alternative to chemical application.

Key words: Biocontrol, *Escherichia coli* O157:H7, *Erwinia carotovora*, lactic acid bacteria, and secondary metabolites.

I. INTRODUCTION

Food safety is one of the major concerns in public health due to outbreaks of food-borne diseases. Consumers and food industries are concerned about the safety of synthetic/chemical preservatives used in food products. As a result, there is increasing demand for natural products that can be used as alternative food preservatives, and lactic acid bacteria (LAB) metabolite is one of such chemical alternatives which can be safely used as food preservative agent (Awah, *et al*, 2018). Contamination and spoilage of ready-to-eat fruits and vegetables by pathogenic microorganisms have been linked to food-borne disease outbreaks globally. The presence of pathogenic bacteria such as *Escherichia coli* O157:H7 and *Erwinia carotovora* in fresh fruits and vegetables is deemed a serious microbial and postharvest hazard (Espigol *et al.*, 2018).

LAB has long history of application in food production such as their use as starter cultures in vegetable fermentation, bakery, dairy and meat production due to their beneficial influence on nutritional, organoleptic, and shelf-life characteristics (Pinar and Yalçin 2015). The biocontrol effect of LAB has been attributed to the production of one or more active secondary metabolites, such as organic acids (lactic, acetic, formic, propionic acids), that intensify their action by reducing the pH of the media, other substances include fatty acids, acetoin, hydrogen peroxide, diacetyl, antifungal compounds (propionate, phenyl-lactate, hydroxyphenyl-lactate, cyclic dipeptides and 3-hydroxy fatty acids), bacteriocins (nisin, reuterin, reutericyclin, pediocin, lacticin, enterocin and others) and bacteriocin-like inhibitory substances-BLIS (Favaro *et. al.*, 2015).

Erwinia carotovora is one of the most important food spoilage bacteria in the food industry which causes soft rot of fruits and vegetables in the field as well as those stored at ambient temperatures. *Escherichia coli* O157:H7 has been classified as one of the most important food contaminants and have been implicated in many cases of food contamination across the world. *E.* coli O157:H7 infections are of particular concern due to the potential severity of symptoms leading to several cases of illness, hospitalization and death. *E. coli* O157:H7 has been recorded as being more transmissible than other *E. coli* serotypes due to its high tolerance to acid, which allows it to easily survive the acidic conditions of the stomach. It also produces shiga toxins, which are heat stable, and

therefore unaffected by conventional food pasteurization methods, thus may cause infection to humans even when they are present in foods in little dose (Rabya *et al.*, 2017).

II. MATERIALS AND METHODS

Location of study:
The study was carried out at the International Institute of Tropical Agriculture (IITA) Ibadan Nigeria.

Collection of samples:
Cassava samples, *Manihot esculenta* (White and yellow tubers) were obtained from the Cassava Programme of the International Institute of Tropical Agriculture, (IITA) Ibadan Nigeria. Suspected diseased samples of fruits and vegetables which include; Pineapples, Avocado pears, Tomatoes, and Cucumbers were randomly purchased from Oje fruits market in Ibadan, Nigeria. Samples were transported immediately to Germplasm Health Import Laboratory of IITA Ibadan for microbiological analysis.

Sample treatment and fermentation:
The cassava samples were peeled, washed and cut into small bits. 500 g of each of the samples was soaked in 750ml of water and allowed to ferment spontaneously for 120 hours.

Isolation, characterization and identification of lactic acid bacteria:
According to (N.C.C.L.S 2004) procedure, 10ml of steep water from the fermenting cassava samples was aseptically taken from each of the fermenting vessel for ten-fold serial dilution at 24hours, 48hours, 72hours, 96hours, and 120hours respectively, and were plated out in the MRS medium (deMann-Rogosa-Sharpe, Oxoid Ltd, Basingstoke, Hampshire, UK), and incubated at 37 ^{o}C for 48 hours using anaerocult gas pack system (Merck, damstadt, Germany). Initial characterization of the isolates included colony and cell morphology, gram staining, KOH reaction, and catalase reaction. Gram positive rods or cocci, KOH positive, catalase negative, oxidase negative and non-motile cells were presumptively identified as LAB. Other biochemical tests such as carbohydrate fermentation, indole and casein hydrolysis test were also carried out. Pure cultures of LAB isolates were stored on slants at 4 ^{o}C for further analysis.

Genomic DNA extraction of the LAB isolates:
Procedure given by Ventura and Zink 2007 was followed, single LAB colonies grown on MRS media were transferred to 1.5 ml of MRS broth, and the cultures were grown on a shaker for 48 hours at 28 ^{o}C. The cultures were centrifuged at 10000 X g for 5 min. The resulting pellets were resuspended in 520µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), while the supernatants were discarded. Fifteen microliters of 20 % SDS and 3 µl of Proteinase K (20 mg/ml) were added and the mixture was incubated for 1 hour at 37 ^{o}C. Then 100 µl of 5 M NaCl and 80 µl of a 10% CTAB solution in 0.7 M NaCl were added and mixed. The suspension was incubated for 10 min at 65^{0} C and kept on ice for 15 min. An equal volume of chloroform: isoamyl alcohol (24:1) was added, followed by incubation on ice for 5 minutes and centrifugation at 7200 x g for 20min. The aqueous phase was transferred to a new tube, isopropanol (1: 0.6) was added and DNA was precipitated at -20 ^{o}C for 16 hours. The DNA of the LAB isolates were collected by centrifugation at 7200 x g for 10 minutes, washed with 500µl of 70% ethanol, air-dried at room temperature for approximately 3 hours and finally dissolved in 50µl of TE buffer.

Polymerase chain reaction (PCR):
Twenty-five microliter (25µl) of each dATP, dCTP, dGTP and dTTP was mixed from a 100mM stock. The final concentration of each dNTP in this mixture was 25 mM. Then 0.508g of $MgCl_2.6H_20$ was dissolved in100 ml of distilled water, sterilized by autoclaving and stored at -20 ^{o}C for further use (Awah *et al.*, 2018).

Primers used:

27F	AGAGTTTGATCMTGGCTCAG
1525r	AAGGAGGTGWTCCARCC

PCR Conditions: 1 cycle of 94 ^{o}C (2 min); 29 cycles of 94 ^{o}C (30s), 50 ^{o}C (1 min), 72 ^{o}C (1.5 min); 1 cycle of 72 ^{o}C (3 min); 4 ^{o}C. Separation of the LAB DNA was carried out in 1% agarose gel.

Preparation secondary metabolite:
The LAB isolates were inoculated into conical flasks containing 100ml MRS broth covered with sterile cotton wool and aluminum foil and clipped unto a wrist action shaker (Burrell Scientific Pittsburgh, P.A. U.S.A) and gently shook for 48 hours at 30 ^{o}C. The secondary metabolite was obtained by centrifuging the broth at 7,000 rpm/rcf for 10 minutes and the supernatant of each isolate was filtrated using sterile filter paper (0.45µm-pore-size filter, Millipore).

Bacterial pathogens:
The two bacterial pathogens (*Erwinia carotovora and E. coli* O157:H7) used for this research were obtained from the culture collection unit of the germplasm health import/export laboratory of IITA, Ibadan Nigeria.

Pathogenicity test:
Pathogenicity test was carried out to determine if the organisms responsible for contamination and spoilage were host specific to the fruits and vegetables used for this research. The procedure described by Agrios (2005) was followed.

Quantitative estimation of diacetyl:
Diacetyl production was determined by transferring 25ml of broth cultures of test organisms into 100ml flasks. Hydroxylamine solution (7.5 ml) of 1 molar was added to the flask and to a similar flask for residual titration. Both flasks were titrated with 0.1 M HCL to a greenish yellow end point using bromothymol blue as indicator. The equivalence factor of HCL to diacetyl is 21.52 mg. The concentration of diacetyl produced was calculated using the A.O.A.C. (2000) procedure.

$$AK = \frac{(B-S)(100E)}{W}$$

Where AK = % of diacetyl
B- S = volume of HCL used
E = equivalence factor (21.52/mg)
W = volume of broth 100 = constant

Quantitative estimation of hydrogen peroxide:
Hydrogen peroxide production was determined by measuring 25ml of broth cultures of the test organisms into a 100ml flask and to this was added 25ml of freshly prepared 0.1M H_2SO_4. It was then titrated with 0.1M potassium permanganate ($KMnO_4$). (Each milliliter of 0.1 N $KMnO_4$ is equivalent to 1.701 mg of H_2O_2). A de-colorization of the sample showed the end point. The volume of H_2O_2 produced was then calculated (A.O.A.C 2000).

$$H_2O_2 \text{ produced} = \frac{\text{ml } KMNO_4 \times NKMNO_4 \times M.E}{\text{ml } H_2SO_4 \text{ X volume of sample}} \times 100$$

Where ML $KMNO_4$ = volume of $KMNO_4$
$NKMNO_4$ = Normality of $KMNO_4$
ml H_2SO_4 = Volume of H_2SO_4 used
M.E = Equivalence factor (1.701/mg)

Quantitative estimation of lactic acid:
The quantity of lactic acid produced by antimicrobial producing LAB isolates was determined by transferring 25ml of broth cultures of test organisms into 100 ml flasks. This was titrated with 0.1M NaOH and 1 ml of phenolphthalein indicator (0.5 % in 5 % alcohol). The titratable acidity was calculated as lactic acid (% w/v). Each milliliter of 1 N NaOH is equivalent to 90.08 mg of lactic acid. The titratable acidity was then calculated as stated in A.O.A.C (2000).

$$\% \text{ acidity} = \frac{\text{ml NaOH} \times \text{MNaOH} \times \text{M.E}}{\text{Volume of sample used}} \times 100$$

Where; ml NaOH = volume of NaOH used
M NaOH = molarity of NaOH
M.E = equivalence factor (90.08/mg)

Production of crude bacteriocin from LAB isolates:
Lactic acid bacteria isolates were propagated in 1000 ml MRS broth (pH 7.0, glucose, 0.25% w/v, peptone, 0.5% w/v) for 48 hrs at 28 ± 2 ^oC under microaerophilic conditions. For extraction of bacteriocin, a cell-free solution was obtained by centrifuging cultures which had been placed in the freezer for one hour at 4,000 rpm for 20 minutes. The culture was adjusted to pH 7.0 followed by filtration of the supernatant with whatman filter paper no1. The supernatant was dialysed for 24 hours at 4 ^oC (Schillinger and Lucke, 2009).

Antibacterial assay:
Determination of inhibitory activity of LAB metabolites on target bacteria using the agar-well diffusion method:

Nutrient agar and EMB agar were prepared, autoclaved at 121 ºC for 15 minutes, allowed to cool to 45 ºC and poured into sterile petri dishes and allowed to solidify. Using a plastic spreader, the target bacteria: *E. coli* O157:H7 and *Erwinia carotovora* were spread over the surface of the plates, and two wells of 8mm per plate were made using cork borer, then 250µl, 500µl and 1000 µl of the metabolites respectively were added to each well in duplicates and the plates incubated at 27 ºC for 48 hours. For control, sterile distilled water was dispensed into the wells. The zones of inhibition were measured using a standard meter rule (Hamadan and Mikolaccik 2004).

Statistical analysis:
The data collected were analyzed using the SAS scientific comprehensive statistical package (SAS/STAT® Software Version 20.0, 2013).

III. RESULTS

A total number of twenty strains of lactic acid bacteria were isolated from fermented cassava samples in this study; ten strains were further characterized and identified. The species identification was authenticated by partial 16S rRNA gene sequencing. The LAB isolates were identified as *L. Pentosus* strains PIS23 and Reyan20, *L. Plantarum* strains PON10014, CTBRBL268 and N3114, *L. Brevis* strain NS25, *L. delbrueckii* strain NS9, *L. Fermentum* strain NS9, *L. lactis* strain NS32 and *L. Mesenteroides* strain NS73. The cultural, morphological and biochemical characterization of the isolates is shown in Table 1a. All the isolates were Gram positive rods and catalase positive. They all hydrolysed casein and were indole positive while motility test was negative. The LAB isolates showed high variability in growth patterns under different concentrations of NaCl. Fifty percent of the isolates tolerated growth in 4.4% and 4.5% NaCl while 60% of the isolates grew in 6.5% NaCl and 80% in 6.6% NaCl concentrations and only 20% of the isolates were able to grow at all the levels of NaCl concentrations. Most of the isolates preferred growth at higher concentrations (6.5 - 6.6%) of NaCl at varying pH of 3.9, 4.0, 4.4, 4.6, 5.5, and 6.2 while none tolerated the pH of 9.6 as their growth was inhibited at this level. The carbohydrate fermentation test is recorded in Table 1b which showed that all the LAB isolates used in this study fermented glucose, lactose, fructose, maltose, salicin and xylose. For growth at different temperatures, most of the isolates tolerated temperatures of 15 ºC, 25 ºC, 37 ºC and 45 ºC as the best growth was recorded at 37 ºC while none tolerated the temperatures of 4 ºC, 10 ºC and 60 ºC respectively as also recorded in our previous study (Awah *et al*., 2018).

Table 1a: Identification of the lactic acid bacterial strains isolated from the cassava samples

LAB I.D	Source	Gram reaction	Shape	Catalase Test	Casein hydrolysis	Indole Test	Motility Test	4.4% NaCl	4.5% NaCl	6.5% NaCl	6.6% NaCl	Probable identity
1	WC	+	R	-	+	+	-	-	-	+	-	*L. pentosus*
2	WC	+	R	-	+	+	-	-	-	+	-	*L. lactis*
3	YC	+	R	-	+	+	-	+	+	+	+	*L. fermentum*
4	YC	+	R	-	+	+	-	+	-	+	+	*L. plantarum*
5	YC	+	R	-	+	+	-	+	+	+	+	*L. lactis*
6	WC	+	R	-	+	+	-	-	+	+	+	*L. delbrueckii*
7	WC	+	R	-	+	+	-	+	+	-	+	*L.brevis*
8	YC	+	R	-	+	+	-	-	+	-	+	*L. plantarum*
9	YC	+	R	-	+	+	-	+	-	+	+	*L. pentosus*
10	YC	+	R	-	+	+	-	-	-	-	+	*L. mesenteroides*

Key: WC= White cassava, YC= Yellow cassava, NaCl= Sodium chloride, + = Positive, - = Negative, Shape →R= Rod.

Table 1b: Identification of the lactic acid bacterial strains isolated from the cassava samples

LAB I.D	Source	Carbohydrate fermentation										Growth at different temperatures (°C)							Growth at different pH								Probable identity
		glu	fru	Suc	man	mal	sor	gal	lac	sal	xyl	4	10	15	25	37	45	60	3.9	4.0	4.4	4.6	5.5	6.2	7.0	9.6	
1	WC	+	+	-	+	+	-	+	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+	-	-	*L. pentosus*
2	WC	+	+	-	-	+	-	+	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+	-	-	*L. lactis*
3	YC	+	+	+	+	+	+	+	-	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+	+	-	*L. fermentum*
4	YC	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+	+	-	*L. plantarum*
5	YC	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+	-	-	*L. lactis*
6	WC	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+	-	-	*L. delbrueckii*
7	WC	+	+	+	+	+	-	+	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+	-	-	*L.brevis*
8	YC	+	+	+	-	+	+	+	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+	-	-	*L. plantarum*
9	YC	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+	-	-	*L. pentosus*
10	YC	+	+	+	-	+	+	+	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+	-	-	*L. mesenteroides*

Sugars: glu=glucose, fru=fructose, suc=sucrose, man=mannitol, sor=sorbitol, gal=galactose, lac=lactose, sal=salicin, xyl=xylose,
+ = Positive, - = Negative.

Probable identity of the LAB isolates based on the overall biochemical reaction and carbohydrate fermentation tests showed the presence of *Lactobacillus pentosus, L. lactis, L. fermentum, L. plantarum, L. delbrueckii, L. brevis* and *L. mesenteroides.* Six suspected isolates showed very clear bands (lanes 3, 6-10) in the gel electrophoresis of the DNA samples using 1500 base pairs ladder as indicated in figure 1.The band in lane 2 was slightly clear while those in lanes 1, 4 and 5 had faint bands. All the bands were above 1000 base pairs hence their selection for further identification. For genotypic identification, the 16S rRNA gene sequences of all the selected isolates were matched with the GenBank Database of NCBI via BLAST and identified as *L. pentosus* strains PIS23 and Reyan20, *L. Plantarum* strains PON10014, CTBRBL268 and N3114, *L. Brevis* strain NS25, *L. delbrueckii* strain NS9, *L. fermentum* strain NS9, *L. lactis* strain NS32 and *L. mesenteroides* strain NS73 as also confirmed from our previous studies (Awah *et al*., 2018).

The diacetyl, hydrogen peroxide and lactic acid concentration of the LAB metabolites are shown in figure 2. *L. plantarum* recorded the highest diacetyl concentration at 3.80 g/L, while *L. brevis* had the lowest at 2.13g/L. *L. plantarum* also recorded the highest hydrogen peroxide and lactic acid concentration at 0.009 g/L and 2.97 g/L respectively, while *L. delbrueckii* recorded the lowest hydrogen peroxide concentration and lactic acid concentration at 0.005 g/L and 2.10 g/L respectively. All the isolates were able to produce crude bacteriocin at different levels (figure: 3). *L. lactis* strain NS32 produced the highest amount of crude bacteriocin at 21.45(IU/ml), while *L.brevis* strain NS25 produced the lowest amount at 15.21(IU/ml).

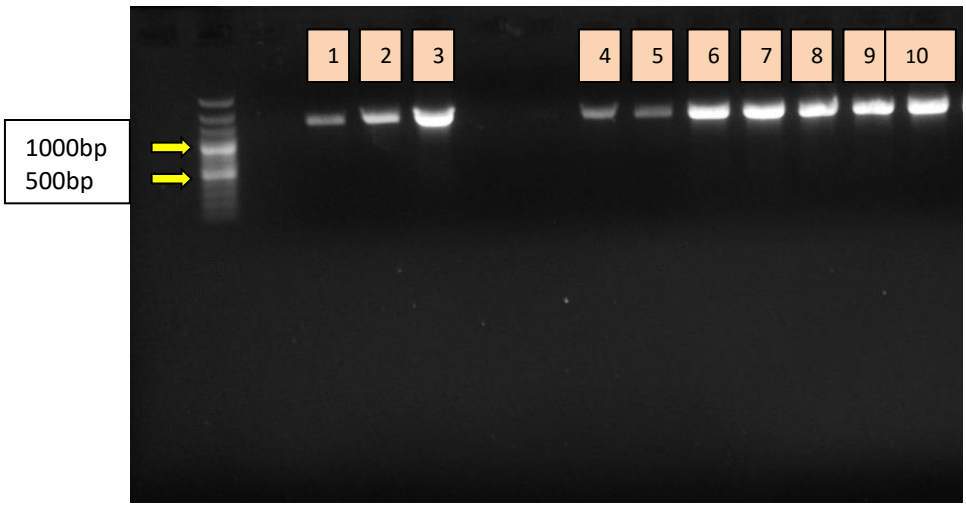


Fig: 1. Gel electrophoresis of the lactic acid bacteria DNA extracts

1. *Lactococcus lactis*
2. *Lactobacillus fermentum*
3. *Lactobacillus Plantarum*
4. *Lactobacillus brevis*
5. *Lactobacillus pentosus*
6. *LactobacillusPlantarum*
7. *LactobacillusPlantarum*
8. *Lactococcus lactis*
9. *Lactobacillus delbrueckii*
10. *Leuconostoc mesenteroides*

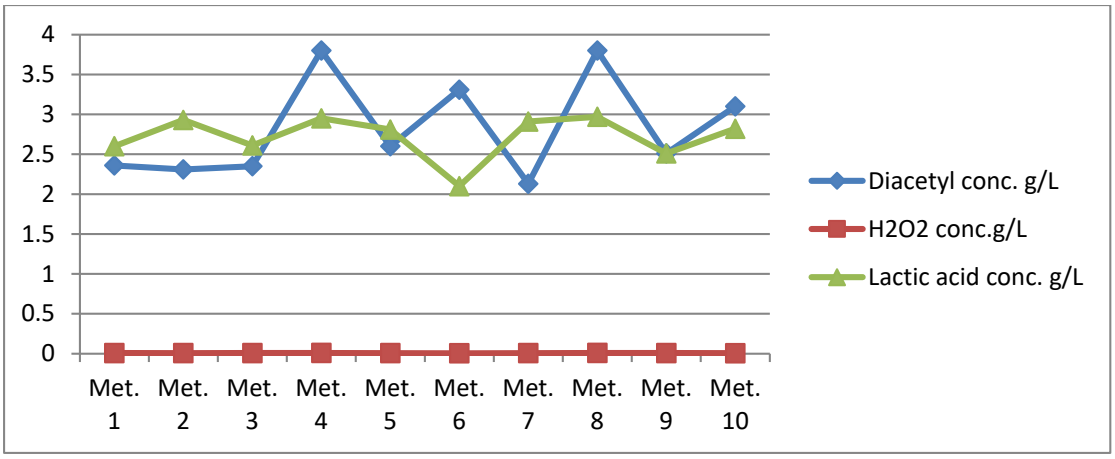


Fig.2. Determination and quantification of diacetyl, hydrogen peroxide and lactic acid concentration in the LAB metabolites.

Sample I.D	Source	LAB species
Met. 1	WC	*L. pentosus* strain PIS23
Met. 2	WC	*L. lactis* strain NS32
Met. 3	YC	*L. fermentum* strain NS9
Met. 4	YC	*L. plantarum* strain N3114
Met. 5	YC	*L. lactis* strain NS32
Met. 6	WC	*L. delbrueckii* strain LB2
Met. 7	WC	*L.brevis* strain NS25
Met. 8	YC	*L. plantarum* strain PON10014
Met. 9	YC	*L. pentosus* strain Reyan 20
Met. 10	YC	*L. mesenteroides* strain NS73

Met. 1-10 = LAB metabolites, YC= Yellow Cassava, WC= White Cassava.

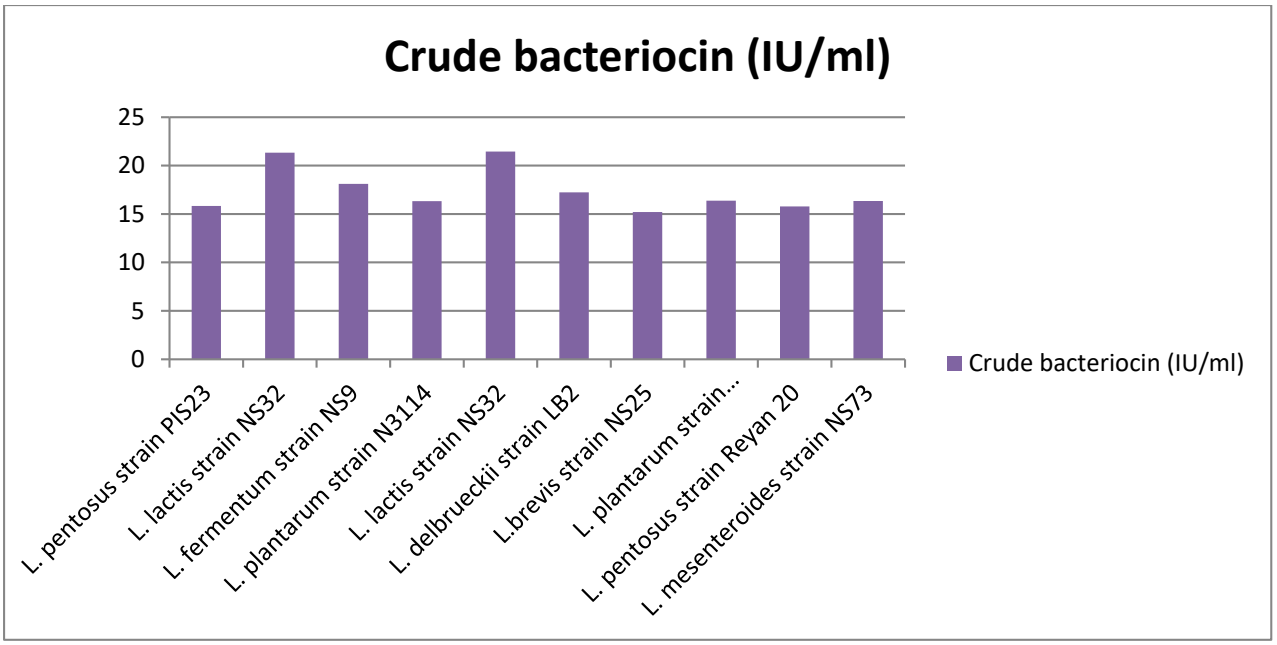


Fig. 3: Quantitative estimation of the amount of crude bacteriocin produced by the LAB isolates.

Table 2 shows the effect of the ten LAB metabolites on the growth and survival of *E. coli* O157:H7. Varying degrees of inhibition was recorded for all concentrations of the metabolites at 24 and 48 hours respectively, while the controls had no zone of inhibition all through the 48 hours of incubation. The recorded zone of inhibition was between 2 mm - 3 mm (2 ± 0.289^{a} - 3 ± 0.289^{b}) at 250µl concentration of the LAB metabolites, 4 mm - 8 mm (4 ± 0.289^{a} - 8 ± 0.289^{d}) at 500µl concentration and 16 mm - 22 mm (16 ± 0.289^{a} - 22 ± 0.289^{f}) at 1000µl concentration. The statistical analysis shows that the inhibitions vary significantly at different levels: *** = significant at P = 0.01, ** = significant at P = 0.05 and ns = not significant.

Table 3 shows the effect of the ten LAB metabolites on the growth and survival of *E. carotovora*. Varying degrees of inhibition was also recorded for all concentrations of the metabolites at 24 and 48 hours respectively, while the controls had no zone of inhibition all through the 48 hours of incubation. The recorded zone of inhibition was between 2 mm - 5 mm (2 ± 0.289^{a} - 5 ± 0.289^{d}) at 250µl concentration of the LAB metabolites, 9 mm - 12 mm (9 ± 0.289^{a} - 12 ± 0.289^{d}) at 500µl concentration and 16 mm - 23 mm (16 ± 0.289^{a} - 23 ± 0.289^{d}) at 1000µl concentration. The statistical analysis shows that the inhibitions vary significantly at different levels: *** = significant at P = 0.01, ** = significant at P = 0.05 and ns = not significant.

Table 2: Effect of LAB metabolites (250µl, 500µl and 1000µl respectively) on the growth of *E. coli* O157:H7 on EMB agar incubated at 27 °C for 48 hours.

Hours	Dilutions (Microliter)	Lactic acid bacteria metabolites										ANOVA F-Statistic
		1	2	3	4	5	6	7	8	9	10	
24	250µl	2±0.289^{a}	3±0.289^{b}	2±0.289^{a}	2±0.289^{a}	2.5±0.289^{ab}	2±0.289^{a}	2±0.289^{a}	2±0.289^{a}	2±0.289^{a}	2±0.289^{a}	1.517^{ns}
	500µl	4±0.289^{a}	5±0.289^{b}	5±0.289^{b}	6±0.289^{c}	7±0.289^{d}	8±0.289^{c}	5±0.289^{b}	6±0.289^{c}	4±0.289^{a}	4±0.289^{a}	12.80***
	1000µl	16±0.289^{a}	16±0.289^{a}	16±0.289^{a}	16±0.289^{a}	19±0.289^{c}	18±0.289^{b}	19±0.289^{c}	21±0.289^{e}	20±0.289^{d}	20±0.289^{d}	45.380***
	Control	0	0	0	0	0	0	0	0	0	0	.000^{ns}
48	250µl	3±0.289^{b}	3±0.289^{b}	2±0.289^{a}	2±0.289^{a}	3±0.289^{b}	2±0.289^{a}	2±0.289^{a}	2±0.289^{a}	2±0.289^{a}	2.5±0.289^{ab}	2.997**
	500µl	5±0.289^{a}	5±0.289^{a}	5±0.289^{a}	7±0.289^{c}	8±0.289^{d}	6±0.289^{b}	5±0.289^{a}	7±0.289^{c}	5±0.289^{a}	5±0.289^{a}	15.467***
	1000µl	16±0.289^{a}	17±0.289^{b}	17±0.289^{b}	16±0.289^{a}	20±0.289^{d}	19±0.289^{c}	19±0.289^{c}	22±0.289^{f}	21±0.289^{e}	21±0.289^{e}	58.133***
	Control	0	0	0	0	0	0	0	0	0	0	.000^{ns}

1-10 = Ten LAB metabolites.

Results are means± standard error of means of three replicates. Values in each row followed by different superscripts within same row are significantly different. *** = significant at P = 0.01 and ** = significant at P = 0.05, ns = not significant.

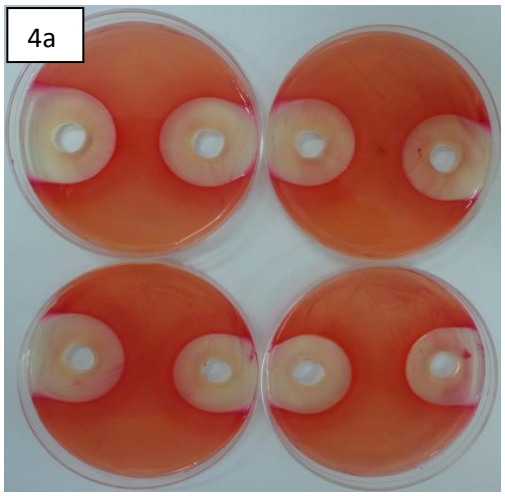
Table 3: Effect of LAB metabolites (250µl, 500µl and 1000µl respectively) on the growth of *E. carotovora* on nutrient agar incubated at 27 °C for 48 hours.

Hours	Dilutions (Microliter)	LACTIC ACID BACTERIA METABOLITES										ANOVA F-Statistic
		1	2	3	4	5	6	7	8	9	10	
24	250µl	2±0.289^{a}	2±0.289^{a}	3±0.289^{b}	3±0.289^{b}	4±0.289^{c}	2±0.289^{a}	2±0.289^{a}	5±0.289^{d}	4±0.289c	5±0.289^{d}	18.133***
	500µl	10±0.289^{b}	10±0.289^{b}	11±0.289^{c}	10±0.289^{b}	11±0.289^{c}	9±0.289^{a}	9±0.289^{a}	12±0.289^{d}	11±0.289c	10±0.289^{b}	10.800***
	1000µl	17±0.289^{b}	16±0.289^{a}	20±0.289^{d}	20±0.289d	23±0.289^{f}	20±0.289^{d}	20±0.289d	23±0.289^{f}	22±0.289e	18±0.289^{c}	67.867***
	Control	0	0	0	0	0	0	0	0	0	0	.000^{ns}
48	250µl	2±0.289^{a}	2±0.289^{a}	3±0.289^{b}	3±0.289^{b}	5±0.289^{d}	2±0.289^{a}	3±0.289^{b}	5±0.289^{d}	4±0.289^{c}	5±0.289^{d}	19.200***
	500µl	11±0.289^{c}	10±0.289^{b}	11±0.289^{c}	10±0.289^{b}	11±0.289^{c}	10±0.289^{b}	9±0.289^{a}	12±0.289^{d}	12±0.289^{d}	10±0.289^{b}	11.200***
	1000µl	17±0.289^{a}	17±0.289^{a}	21±0.289^{c}	21±0.289^{c}	23±0.289^{d}	20±0.289^{b}	21±0.289^{c}	23±0.289^{d}	23±0.289^{d}	20±0.289^{b}	59.200***
	Control	0	0	0	0	0	0	0	0	0	0	.000^{ns}

1-10 = Ten LAB metabolites.

Results are means± standard error of means of three replicates. Values in each row followed by different superscript within same row are significantly different. *** = significant at P = 0.01. ns = not significant.

Figures 4 and 5 show the inhibitory activities of the LAB metabolites against the growth of *Erwinia carotovora* via the agar-well diffusion test on nutrient agar incubated at 27 ^{o}C for 48 hours and *E.coli* O157:H7 on EMB agar incubated at 27 ^{o}C for 48 hours with their respective controls.



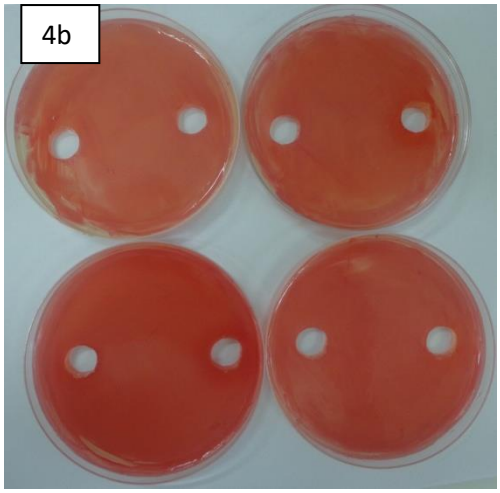
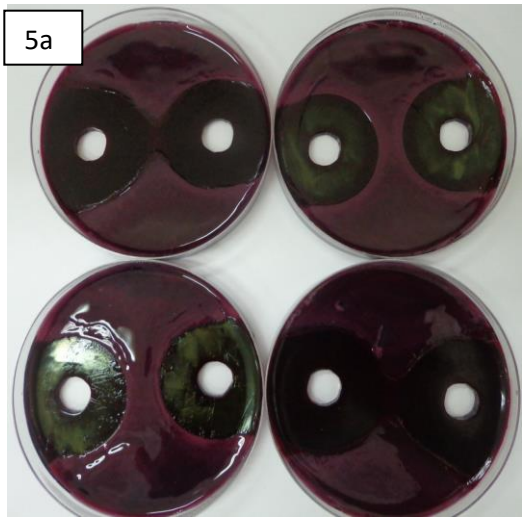


Fig. 4a: Zones of inhibition of LAB metabolites against *E. carotovora* on nutrient agar incubated at 27 ^{o}C for 48 hours. **Fig. 4b:** (Control).



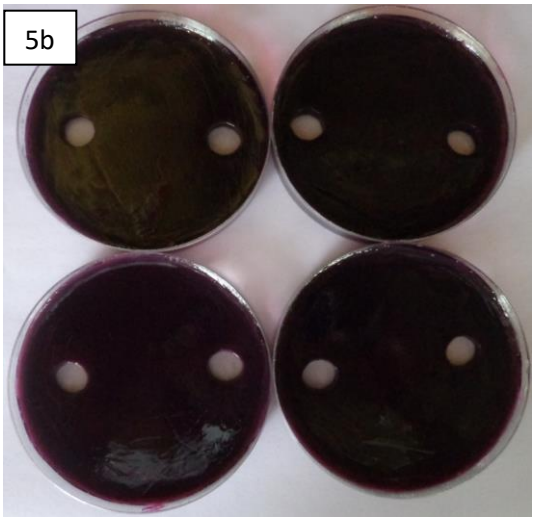


Fig. 5a: Zones of inhibition of LAB metabolites against *E.coli* O157:H7 on EMB agar incubated at 27 ^{o}C for 48 hours. **Fig. 5b:** (Control).

Figures 6 and 7 below show the preliminary study of directly applying the LAB metabolites on fresh fruits and vegetables for inhibition of the growth or appearance of bacterial pathogens. At the sixth day, the fruits and vegetables without the LAB metabolite treatment started showing signs of bacterial growth while those treated with LAB metabolites showed no sign of bacterial appearance or growth. At the fourteenth day there was full evidence of the presence of the bacterial pathogens on the samples without the LAB metabolite treatment while those treated with the metabolites started showing signs of bacterial growth at the twentieth (20th) day.

Fig. 6a: LAB metabolite-treated and non-treated tomatoes at the 10th day of preliminary test.

Fig. 6b: Non-treated tomatoes at the 14th day of preliminary test (Control).

Fig. 7a. LAB metabolite-treated avocado pears at 20th day of the preliminary test.
Fig. 7b. Non-treated avocado pears at 20th day of the preliminary test (control).

IV. DISCUSSION

Bacterial spoilage and contamination of fresh fruits and vegetables constitute major agricultural and health concerns to both producers and consumers worldwide. Lactic acid bacteria and their secondary metabolites have been employed for centuries in the preservation of food and milk products and thus generally recognized as safe for human consumption. Twenty LAB isolates were obtained from the fermented cassava samples used in this study and ten were further characterized and identified. The species identification was authenticated by partial 16S rRNA gene sequencing. The LAB isolates were identified as *L. Pentosus* strains PIS23 and Reyan20, *L. Plantarum* strains PON10014, CTBRBL268 and N3114, *L. Brevis* strain NS25, *L. delbrueckii* strain NS9, *L. Fermentum* strain NS9, *L. lactis* strain NS32 and *L. Mesenteroides* strain NS73. Molecular identification of the lactic acid bacterial strains which produced the metabolites used in this study demonstrated the dominance of *L. Pentosus*, *L. Plantarum*, *L. brevis*, *L. delbrueckii*, *L. fermentum*, *L. lactis* and *L. mesenteroides* in fermented cassava. These identified LAB strains were in accordance with those earlier identified from similar traditional fermented food products (Adeyemo *et al.*, 2018; Monika *et al.*, 2017; Omemu, and Faniran, 2011).

The LAB isolates used in this study produced different antimicrobial compounds which include: lactic acid, hydrogen peroxide, diacetyl and crude bacteriocin. The production of antimicrobial compounds by these LAB strains confers on them the potential ability to be used as biocontrol agents and sanitizers in the food industry. The growth and survival of the two target bacteria: *E.coli* O157:H7 and *Erwinia carotovora* used in this study were strongly inhibited by seven out of the ten LAB metabolites at varying degrees which include met 1, 2, 3, 5, 7, 8 and 9 produced by *L. pentosus* strain PIS 23, *L. Lactis* strain NS 32, *L. fermentum* strain NS9, *L. brevis strain* NS 25, *L. plantarum* strain PON10014 and *L. pentosus* strain Reyan 20. Similar to this finding, Adeyemo *et al.*, (2018) observed varying degrees of inhibition on the growth of various food borne pathogens by secondary metabolites of lactic acid bacteria. Awojobi *et al.*, (2016) reported the inhibition of the growth of pathogenic and spoilage microorganisms in fresh pineapple juice by direct inoculation of lactic acid bacteria isolates. Antimicrobial compounds have the ability to antagonize the growth of some spoilage

and pathogenic bacteria in foods and food products and have been explored in the control of most unwanted microorganisms (Garcha and Rani 2018). Lactic acid bacteria are known to produce secondary metabolites such as lactic acids which exert their antimicrobial effect by interfering with cell membrane activity, reducing intracellular pH and inhibiting various metabolic functions in the cell of microbes, therefore each antimicrobial compound produced during fermentation of foods provides an additional hurdle for food-spoilage and other pathogenic bacteria to overcome before they can survive or proliferate in such foods (Şanlıbaba and Güçer 2015).

All the isolates were able to produce crude bacteriocin at different levels. Many lactic acid bacteria strains isolated from various fermented foods have been shown to be producers of bacteriocins and are capable of inhibiting the growth of spoilage bacteria and food borne pathogens such as *Listeria monocytogenes, E. carotovora, Pseudomonas aeruginosa, Staphylococcus aureus*, *Salmonella typhimoreum*, and *E. coli* O157:H7 (Islam *et al*., 2020; Garcha and Rani 2018; Adeyemo *et al.*, 2018).
The Pathogenicity test showed that each infected fruit and vegetable used in this study gave the initial organism that caused their contamination and spoilage. Amongst the two target bacteria used in this study, *E. coli* O157:H7 was highly pathogenic leading to rapid disintegration of the infected fruits and vegetables in 3-5 days while *E. Carotovora* was moderately pathogenic but also caused soft rot on the infected fruits and vegetables. However, the results obtained form the study on applying the LAB metabolites on fresh fruits and vegetables for inhibition of growth or appearance of the target bacterial pathogens showed that the metabolites have the capacity to inhibit the growth and survival of the these bacteria. A common strategy for preservation of foods that are eaten raw or without further cooking such as fruits and vegetables is the application of edible films or coatings containing antimicrobial substances. The incorporation of antimicrobial compounds such as bacteriocins, nisin, lactic acid, diacetyl etc is an interesting alternative for ensuring the control of pathogenic microorganisms in fresh and raw food products (Valdés *et al.*, 2017).

V. CONCLUSION

The rise in food borne diseases as a result of the consumption of fresh fruits and vegetables contaminated by pathogenic bacteria is a big health concern in developing countries. However, the growth of pathogenic bacteria in the fruits and vegetables used for this study were inhibited by the LAB metabolites, which also played significant role in extending the shelf life thus, secondary metabolites isolated from lactic acid bacteria can be employed as sanitizers and biocontrol agents in food processing to serve as alternative to chemical preservatives or additives used in food preservation.

Conflicts of Interest:
The authors hereby declare no potential conflicts of interest with respect to this research work.

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