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Bio-preservative potential of lactic acid bacteria metabolites against fungal pathogens

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Bio-preservative potential of the secondary metabolites produced by lactic acid bacteria (LAB) isolated from fermented cassava was assessed against fungal pathogens associated with spoilage of fresh fruits and vegetables. Twenty LAB isolates were identified according to standard morphological and biochemical methods and ten were subjected to phenotypic and genotypic identifications. The metabolites produced were tested for anti-fungal activity using agar-dilution and agar-well diffusion methods. The metabolites were used as sanitizers and biopreservatives by applying them on fresh fruits and vegetables for inhibition of the growth of spoilage fungi and extension of shelf-life. 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. When fresh fruits and vegetables were inoculated with the metabolites, there was strong inhibition of the radial growth and spores of the fungal pathogens. This study shows that metabolites from fermented cassava are a good source of lactic acid bacteria with the ability to inhibit wide range of spoilage fungi, and can be employed in prolonging the shelf-life of fresh fruits and vegetables.

Key words: Lactic acid bacteria, metabolites, biopreservation, *Penicillium oxalicum, Fusarium verticillioides, Aspergillus niger*.

INTRODUCTION

Microbial spoilage of food is one of the major concerns of food industries as it leads to economic losses. It is also a serious problem worldwide. Therefore, consumption of such spoilt food by humans can cause food-borne infections and intoxications due to the presence of microbial pathogens such as *Staphylococcus aureus*, entero-pathogenic *Escherichia coli*, *Shigella dysenteriae* and their toxins. Thus, it is extremely important to monitor various factors from the production of the food and its final distribution to the consumers to prevent food spoilage (Priyanka et al., 2016).

The use of microorganisms such as lactic acid bacteria

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(LAB) and their metabolites to preserve food has gained importance in recent years due to the demand for reduced use of chemical preservatives by consumers and the increasing number of microbial species resistant to antibiotics and preservatives. Lactic acid bacteria not only produce various antimicrobial compounds that are considered important in the biopreservation of food, but are also cost-effective and safe for human consumption (Wei and HaiKuan, 2014).

Biopreservation of foods through the use of LAB enhances the quality of food due to the production of secondary metabolites such as lactic acid, bacteriocin etc which results in decrease of pH. Many lactic acid bacteria are capable of inhibiting the growth of wide variety of food spoilage organisms and the mechanisms exploited in preservation is their potency to produce inhibiting agents like nisin (Hitendra et al., 2016).

Lactic acid bacteria can be added to foods as cultures and they are generally considered to be harmless or even have advantage on human health and are recognized as generally regarded as safe (GRAS) (Hitendra et al., 2016).

In the present study, various strains of lactic acid bacteria were isolated from fermented cassava and their metabolites assessed for antifungal activity against target fungal pathogens associated with spoilage of fresh fruits and vegetables. Direct application of the LAB metabolites on fresh fruits and vegetables as biopreservative agent was also demonstrated.

MATERIALS AND METHODS

Collection of samples

Cassava samples [*Manihot esculenta* (white and yellow roots)] 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, peppers, 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 (white and yellow roots) were peeled, washed and cut into small bits. 500 g of each of the samples was soaked in 750 mL of water and allowed to ferment spontaneously for 120 h.

Isolation and identification of lactic acid bacteria

According to NCCLS (2004) procedure, 10 mL of steep water from the fermenting samples was aseptically taken from each of the fermentation vessel at 24, 48, 72, 96 and 120 h respectively, and plated out in MRS medium, Oxoid Ltd, Basingstoke, Hampshire, UK); and incubated at 37°C for 48 h 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. Presumptive LAB isolates were further tested for carbohydrate fermentation, indole and casein hydrolysis. Isolates were subjected to growth under 4.4, 4.5, 6.5 and 6.6% NaCl concentrations. Growth at different temperatures and pH were also determined. Pure cultures of LAB isolates were stored at 4°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 incubator for 48 h at 28 ^{o}C. DNA of the LAB isolates was collected by centrifugation at 7200 x g. Quantification of the nucleic acid concentration and purity of the DNA extract were done using Nanodrop (2000) Spectrophotometer connected to a computer.

DNA selection by polymerase chain reaction (PCR)

Using the Taq DNA polymerase 25 µl of each dATP, dCTP, dGTP and dTTP was mixed from a 100 mM stock. The final concentration of each dNTP in this mixture was 25 mM. Then 0.508 g of $MgCl_2.6H_2O$ was dissolved in100 mL of distilled water, sterilized by autoclaving and stored at -20°C.
Primers (Sigma-Aldrich); 0.35 µL each was used:

27F: AGAGTTTGATCMTGGCTCAG
1525r: AAGGAGGTGWTCCARCC

PCR Conditions

1 cycle of 94°C (2 min); 29 cycles of 94°C (30s), 50°C (1 min), 72°C (1.5 min); 1 cycle of 72°C (3 min); 4°C using 10X PCR buffer 17.5 µL . Separation of the LAB DNA was carried out in 1% agarose gel and photograph of the gel was taken using an orange filter fitted camera.

Preparation of cell free supernatant

The LAB isolates were inoculated into conical flasks containing 100 mL MRS broth; they were 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 shaken for 48 h at 30°C. The cell free supernatant was prepared by centrifuging the broth at 7,000 rpm for 10 min and the supernatant of each isolate was filtered using sterile filter paper (0.45 µm-pore-size filter, Millipore).

Isolation and identification of fungal pathogens

Fungal isolates were obtained from the spoilt fruits. Identification was effected by wet- mounting the fungal mycelium with lactophenol-cotton blue and observed under 40X objective lens of the phase contrast microscope. Colony colour, growth pattern on plates, details of philiades and spores were also used as identification parameters with reference to Agrios (2005).

Pathogenicity test

Pathogenicity test was carried out to determine if the organisms responsible for spoilage were host specific to the fruits used for this

research. The procedure described by Agrios (2005) was basically followed.

Quantitative estimation of diacetyl

Diacetyl production was determined by transferring 25 mL of broth cultures of test organisms into 100 mL 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 concentration of diacetyl produced was calculated using the AOAC (1990) procedure.

Quantitative estimation of hydrogen peroxide

Hydrogen peroxide production by the LAB isolates was determined, and the volume produced was then calculated (AOAC, 1990).

Quantitative estimation of lactic acid

The quantity of lactic acid produced by the LAB isolates was determined, and the titratable acidity was then calculated as stated in AOAC (1990).

Production of crude bacteriocin from LAB isolates

Lactic acid bacteria isolates were propagated in 1000 mL MRS broth for 48 h at 28 ± 2°C 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 min. The culture was adjusted to pH 7.0 by means of 1M NaOH to exclude the antimicrobial effect of organic acid, followed by filtration of the supernatant with whatman filter paper no1. The supernatant was dialysed for 24 h at 4°C (Schillinger and Lucke, 2009).

Preparation of the fungal spores

The three fungal isolates*: Penicillium oxalicum, Fusarium verticillioides* and *Aspergillus niger* were inoculated in sterile conical flasks containing 100 mL of potato dextrose broth (PDB); they were 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 shaken for 48 h at 30°C. Then the fungal spores of each isolate were obtained by filtering with sterile filter paper (0.45 µm-pore-size filter, Millipore), (Avis and Belanger, 2001).

Antifungal assay

Determination of inhibitory activity of LAB metabolites on fungal growth using the agar-dilution method

Five and ten milliliter (5 mL and 10 mL) each of the LAB metabolites was dispensed into different sterile Petri dishes in triplicates; 40 mL of Nutrient Broth Yeast (NBY) agar was poured onto it and rocked gently for even distribution of the metabolites, and allowed to solidify. Then the pathogens: *P. oxalicum, F. verticillioides,* and *A. niger* were bored out from their original fully grown plates using 6 mm cork borer and carefully cultured at the center of the plates and incubated at 27°C for six days. For the control, no metabolite was used. Radial growths of the pathogens were measured using standard meter rule (Avis and Belanger, 2001).

Determination of inhibitory activity of LAB metabolites on fungal spore germination by agar-well diffusion method

To determine the inhibitory activity of the LAB metabolites against fungal spore germination, 10^6 conidia/mL of the three fungal spores*: P. oxalicum, F. verticillioides* and *A. niger* respectively were dispensed into different sterile Petri dishes; 40 mL of NBY agar was poured unto it and rocked gently for even distribution of the spores and allowed to solidify. Then, two wells of 8 mm per plate were made using cork borer and 20 µl of MRS agar was dispensed to cover the base of the wells to avoid leaking of the metabolites; 250, 500, and 1000 µl of the metabolites respectively were added to each well in duplicates and the plates incubated at 27°C for 24, 48, and 72 h. For the control, sterile distilled water was dispensed into the wells and incubated at 27°C for 24, 48, and 72 h. The zones of inhibition were measured using a standard meter rule (Schnurer and Magnusson, 2005).

Preliminary study of applying the LAB metabolites

A preliminary study of applying the LAB metabolites on fresh fruits and vegetables (Avocado pear, Pineapple, Cucumber and Tomatoes) for inhibition of the growth of spoilage fungi was carried out. Pin-holes were made on the fruits and vegetables in duplicates using sterile needles and divided into two parts; one was soaked inside the LAB metabolites for 20 min, allowed to air-dry under the Laminar flow sheet while the other part was left without the LAB metabolites. Then fungal spores (10^6 conidia/mL) obtained via the preparation of fungal inoculums of the three target fungi were applied onto the surface of the fruits and vegetables using a sterile plastic spreader. It was wrapped loosely with sterile aluminum foils and kept at ambient temperature for 21 days (Simonne et al., 2004).

Statistical analysis

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

RESULTS

A total of twenty LAB isolates from fermented cassava samples were identified according to standard morphological and biochemical methods and ten were further subjected to phenotypic and genotypic identification. The cultural, morphological and biochemical characterization of the isolates is shown in Table 1a. All the isolates from cassava samples were Gram positive rods and catalase positive. They all hydrolysed casein and were indole positive while motility was negative. The isolates showed high variability in growth patterns under different concentrations of NaCl. Fifty percent of 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. Only 20% of the isolates had growth at all the levels of NaCl concentrations under study. Most of the isolates preferred growth at higher concentrations (6.5-6.6%) of NaCl. The carbohydrate fermentation test in Table 1b showed that all the LAB isolates used in this study fermented glucose, lactose, fructose, maltose,

Table 1a. Identification of lactic acid bacterial isolates from 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*

WC= White cassava; YC= Yellow cassava; NaCl= Sodium chloride; + = Positive; **-** = Negative; Shape→R**=** Rod.

Table 1b. Identification of lactic acid bacterial isolates from 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; mal = maltose; sor = sorbitol; gal = galactose; lac = lactose; sal = salicin; xyl = xylose; + = Positive; - = Negative.

galactose, salicin and xylose. A good number of the isolates tolerated growth temperatures of 15, 25, 37 and 45°C with optimum growth temperature recorded at 37°C. Growth was inhibited at temperatures of 4, 10 and 60°C respectively. The effect of pH on the growth of the isolates showed that the pH levels of 3.9, 4.0, 4.4, 4.6, 5.5, and 6.2 favoured growth but were inhibited by the pH of 7.0 to 9.6. Probable identity of the isolates based on the overall biochemical reaction and carbohydrate fermentation tests showed the presence of *Lactobacillus pentosus, L. lactis,*

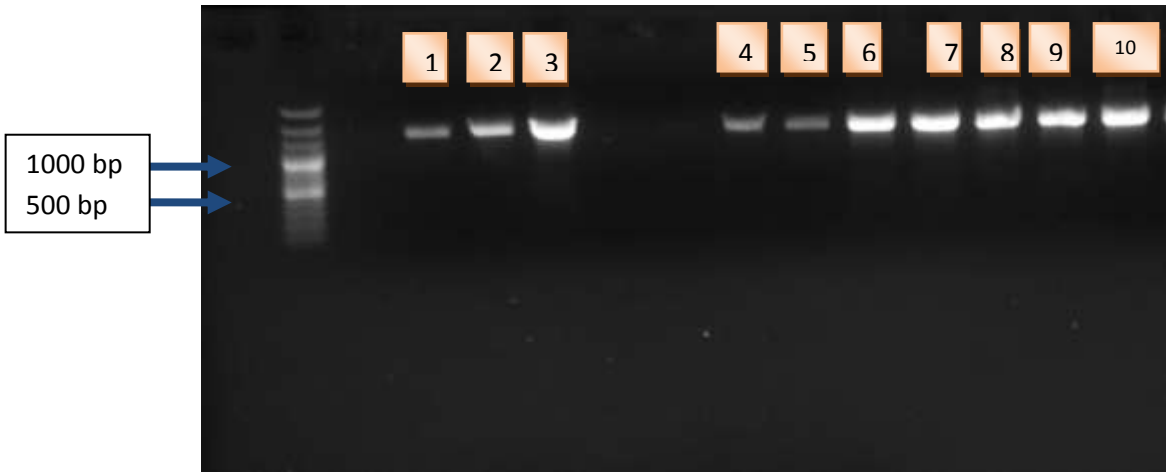


Figure 1. Gel electrophoresis of the lactic acid bacteria DNA extracts. 1, *Lactococcus lactis*; 2, *Lactobacillus fermentum*; 3, *Lactobacillus plantarum;* 4, *Lactobacillus brevis*; 5, *Lactobacillus pentosus*; 6, *Lactobacillus plantarum*; 7, *Lactobacillus plantarum*; 8, *Lactococcus lactis*; 9, *Lactobacillus delbrueckii*; 10, *Leuconostoc mesenteroides.*

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

Sample I.D	Source	LAB species	Diacetyl conc. g/L	H_2O_2 conc. g/L	Lactic acid conc. g/L
Met. 1	WC	*L. pentosus* strain PIS23	2.36	0.008	2.60
Met. 2	WC	*L. lactis* strain NS32	2.31	0.007	2.93
Met. 3	YC	*L. fermentum* strain NS9	2.35	0.008	2.61
Met. 4	YC	*L. plantarum* strain N3114	3.80	0.009	2.95
Met. 5	YC	*L. lactis* strain NS32	2.60	0.007	2.81
Met. 6	WC	*L. delbrueckii* strain LB2	3.31	0.005	2.10
Met. 7	WC	*L.brevis* strain NS25	2.13	0.007	2.91
Met. 8	YC	*L. plantarum* strain PON10014	3.80	0.009	2.97
Met. 9	YC	*L. pentosus* strain Reyan 20	2.51	0.008	2.51
Met. 10	YC	*L. mesenteroides* strain NS73	3.10	0.006	2.82

Met. 1-10 = LAB metabolites, YC= yellow cassava, WC= white cassava.

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 bands were above 1000 base pairs hence were selected for further identification. The band on the gel electrophoresis of the isolate in lane 2 was slightly clear but those in lanes 1, 4 and 5 had no clear bands. For genotypic identification, the 16S rRNA gene sequences of 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. The diacetyl, hydrogen peroxide and lactic acid concentration of the LAB metabolites are shown in Table 2. *L. plantarum* recorded the highest diacetyl concentration at 3.80 g/L, while *L.brevis* had the lowest at 2.13 g/L. *L. plantarum* also recorded the highest hydrogen peroxide and lactic acid concentration at 0.009 and 2.97 g/L respectively, while *L. delbrueckii* recorded the lowest hydrogen peroxide concentration and lactic acid concentration at 0.005 and 2.10 g/L respectively. The source of the LAB did not play any significant role under this condition. All the isolates were able to produce crude bacteriocin at different levels ranging from 15.21 – 21.45 IU/mL (Table 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. Varying degrees of inhibition were detected against the fungi pathogens used in this study *via* the antifungal assay tested with the LAB metabolites as shown in Figure 2. The LAB metabolites

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

LAB species	Crude bacteriocin (IU/mL)
L. pentosus strain PIS23	15.83
L. lactis strain NS32	21.33
L. fermentum strain NS9	18.12
L. plantarum strain N3114	16.33
L. lactis strain NS32	21.45
L. delbrueckii strain LB2	17.23
L. brevis strain NS25	15.21
L. plantarum strain PON10014	16.38
L. pentosus strain Reyan 20	15.78
L. mesenteroides strain NS73	16.35

Incubation at 144 hours Control

Figure 2. Effect of LAB metabolites on the growth of *Penicillium oxalicum* in NBY agar incubated at 27°C for 144 h.

strongly inhibited the radial growth and spore germination of *P. oxalicum*, and *F. verticillioides* but there was weak inhibition against *A. niger*. Table 4 quantitatively shows the effect of the LAB metabolites against radial growth of *P. oxalicum*. There were significant differences in the potency of the LAB metabolites against the fungal pathogens. Both the length of incubation and the metabolite concentration also significantly affected the inhibition of the radial growth of *P. oxalicum.* Metabolite 5 produced by *L. lactis* strain NS32 recorded the highest inhibition of the radial growth at 144 h giving 7 (7 ± 0.289) and 5 mm (5 ± 0.289) inhibition respectively at 5 and 10 mL concentration. Figure 3 shows the effect of LAB metabolites against radial growth of *Aspergillus niger* on NBY agar incubated at 27°C for 144 h. The effects of LAB metabolites on the spore germination of *Aspergillus niger* shown in Table 5 indicate that the metabolites that recorded the highest inhibitions at 144 h were metabolites 5 (18 mm) and 10 (18.5 mm) produced by *L. lactis* strain NS32 and *L. mesenteroides* strain NS73 respectively at 1000 µl of concentration, followed by metabolite 8 (17.5 mm) and 7 (14.5 mm) produced by *L. plantarum* strain PON10014 and *L.brevis* strain NS25 respectively. Time of incubation and the concentration of the metabolites significantly affected the rate of fungal spore growth inhibition. No spore inhibitions were recorded within 24 h of growth for all concentrations of LAB metabolites. The rate of inhibitions increased with increase in metabolite concentrations and the length of incubation. All the control samples did not record any level of inhibitions. Table 6 shows the effect of the LAB metabolites against spore germination of *Fusarium verticilliodes.* Inhibition of spore germination was significantly affected by the source and the concentration of the metabolite as well as the length of incubation. There was varying degrees of inhibition of the metabolites against the survival of the pathogen. The metabolite that recorded the highest inhibition on spore germination of *Fusarium verticilliodes* at 72 h was metabolite 5 produced by *L. lactis* strain NS32 which gave 23 mm at 1000 µl of concentration, followed by metabolite 1 (22 mm) and 7 (21 mm) produced by *L. pentosus* strain PIS23 and *L. brevis* strain NS25 respectively. When the LAB metabolites were applied on fresh fruits and vegetables used in this study (Avocado pear, Pineapple, Cucumber and Tomatoes) for inhibition of growth or appearance of the target spoilage fungi, they were in good condition over a period of 21 days before been challenged by the spoilage fungi as compared to the control.

DISCUSSION

Lactic acid bacteria have been employed for centuries in the preservation of food and milk products and thus acquired the GRAS (generally recognized as safe) status. A total number of twenty strains of LAB were isolated from 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 LAB isolates used in this study produced different antifungal compounds via the secondary metabolites. The morphological, biochemical, physiological and genotypic characterization of the isolates revealed that *L. plantarum* produced lactic acid, diacetyl and hydrogen peroxide in abundance. The prominent production of lactic acid by the LABs caused a reduction in the pH which can inhibit the growth of many pathogens. The lactic acid production and the acidity that resulted was important but it was not the sole antimicrobial mechanism; hence it was complemented by other mechanisms such as the production of hydrogen sulphide, synthesis of bacteriocins and possibly other unidentified compounds.

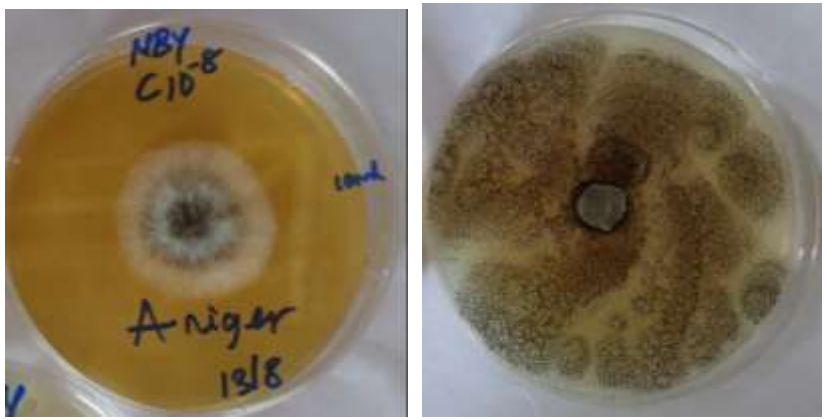
Table 4. Effect of LAB metabolites on radial growth of *P. oxalicum* in NBY agar incubated at 27 °C for 144 h.

Hours	Dilutions (milliliter)	Lactic acid bacteria metabolites										ANOVA F-Statistic
		1	2	3	4	5	6	7	8	9	10	
24	5 mL	5±0.289^{a}	5±0.289^{a}	5±0.289^{a}	5±0.289^{a}	5±0.289^{a}	5±0.289^{a}	5±0.289^{a}	5±0.289^{a}	5±0.289^{a}	5±0.289^{a}	0.000^{ns}
	10 mL	5±0.289^{a}	5±0.289^{a}	5±0.289^{a}	5±0.289^{a}	5±0.289^{a}	5±0.289^{a}	5±0.289^{a}	5±0.289^{a}	5±0.289^{a}	5±0.289^{a}	0.000^{ns}
	Control	7±0.289^{a}	8±0.289^{b}	9±0.289^{c}	7±0.289^{a}	8±0.289^{b}	9±0.289^{c}	8±0.289^{b}	7±0.289^{a}	7±0.289^{a}	9±0.289^{c}	9.200***
48	5 mL	5±0.289^{a}	5±0.289^{a}	6±0.289^{b}	6±0.289^{b}	5±0.289^{a}	6±0.289^{b}	8±0.289^{d}	7±0.289^{c}	9±0.289^{e}	6±0.289^{b}	21.467***
	10 mL	5±0.289^{a}	5±0.289^{a}	5±0.289^{a}	5±0.289^{a}	5±0.289^{a}	5±0.289^{a}	6±0.289^{b}	5±0.289^{a}	6±0.289^{b}	5±0.289^{a}	2.133*
	Control	13±0.289^{e}	15±0.289^{c}	14±0.289^{d}	14±0.289^{d}	14±0.289^{d}	16±0.289^{b}	13±0.289^{e}	13±0.289^{e}	12±0.289^{f}	17±0.289^{a}	27.867***
72	5 mL	10±0.289^{d}	6±0.289^{b}	9±0.289^{c}	10±0.289^{d}	5±0.289^{a}	10±0.289^{d}	10±0.289^{d}	11±0.289^{e}	12±0.289^{f}	10±0.289^{d}	56.133***
	10 mL	7±0.289^{c}	5±0.289^{a}	6±0.289^{b}	7±0.289^{c}	5±0.289^{a}	7±0.289^{c}	8±0.289^{d}	7±0.289^{c}	8±0.289^{d}	7±0.289^{c}	13.467***
	Control	26±0.289^{a}	27±0.289^{a}	28±0.289^{a}	29±0.289^{a}	26±0.289^{a}	33±0.289^{b}	27±0.289^{a}	25±0.289^{a}	26±0.289^{a}	35±0.289^{c}	2.095*
96	5 mL	13±0.289^{e}	7±0.289^{b}	12±0.289^{d}	11±0.289^{c}	5±0.289^{a}	11±0.289^{c}	12±0.289^{d}	13±0.289^{e}	14±0.289^{f}	12±0.289^{d}	96.000***
	10 mL	9±0.289^{d}	6±0.289^{b}	8±0.289^{c}	9±0.289^{d}	5±0.289^{a}	8±0.289^{c}	10±0.289^{e}	9±0.289^{d}	10±0.289^{e}	9±0.289^{d}	32.133***
	Control	38±0.289^{b}	37.5±0.289^{a}	39.5±0.289^{c}	38±0.289^{b}	38±0.289^{b}	40±0.289^{d}	38±0.289^{b}	37±0.289^{a}	39±0.289^{c}	41±0.289^{e}	23.109***
120	5 mL	14±0.289^{e}	8±0.289^{b}	12±0.289^{c}	12±0.289^{c}	6±0.289^{a}	13±0.289^{d}	12±0.289^{c}	14±0.289^{e}	14±0.289^{e}	13±0.289^{d}	87.467***
	10 mL	10±0.289^{c}	6±0.289^{b}	10±0.289^{c}	11±0.289^{d}	5±0.289^{a}	10±0.289^{c}	11±0.289^{d}	10±0.289^{c}	11±0.289^{d}	10±0.289^{c}	53.867***
	Control	42±0.289^{a}	42±0.289^{a}	42±0.289^{a}	42.5±.289^{a}	42.5±.289^{a}	42.5±.289^{a}	42.5±.289^{a}	42±0.289^{a}	42±0.289^{a}	42±0.289^{a}	1.325^{ns}
144	5 mL	15±0.289^{f}	9±0.289^{b}	12±0.289^{c}	13±0.289^{d}	7±0.289^{a}	13±0.289^{d}	14±0.289^{e}	14±0.289^{e}	14±0.289^{e}	13±0.289^{d}	75.200***
	10 mL	11±0.289^{d}	7±0.289^{b}	10±0.289^{c}	11±0.289^{d}	5±0.289^{a}	11±0.289^{d}	11±0.289^{d}	10±0.289^{c}	11±0.289^{d}	10±0.289^{c}	50.800***
	Control	42.5±0.289^{a}	42.5±0.289^{a}	42.5±0.289^{a}	42.5±.289^{a}	42.5±.289^{a}	42.5±.289^{a}	42.5±.289^{a}	42.5±.289^{a}	42.5±.289^{a}	42.5±.289^{a}	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 = 0.1. ns = not significant.

Hydrogen peroxide is an antimicrobial factor. When associated with the lactoperoxidase/ thiocyanate system, hydrogen peroxide leads to the formation of inhibitory compounds which are bacteriostatic. In a recent study by Adss et al. (2017), treatment of tomato fruits with salicylic acid and hydrogen peroxide elicitors enhanced the resistance to fruit rot caused by *Alternaria solani* and decreased the development of post-harvesting fruit rot disease. All the isolates were able to produce crude bacteriocin at different levels. LAB has long been used in a variety of food fermentations by converting lactose to lactic acid, as well as producing additional antimicrobial molecules such as bacteriocins, organic acids, diacetyl, acetoin, hydrogen peroxide and antifungal peptides (Egan et al., 2016).

The study on applying the LAB metabolites on fresh fruits and vegetables for inhibition of growth or appearance of the target spoilage fungi showed that the metabolites have the capacity to be used as biopreservative agent. A common strategy for preservation of foods that are eaten raw or without further cooking is the



Incubation at 144 hours Control

Figure 3. Effect of LAB metabolites on the growth of *Aspergillus niger* in NBY agar incubated at 27°C for 144 h.

Table 5. Effect of LAB metabolites (250, 500 and 1000 µl respectively) on Spore germination of *Aspergillus niger* in NBY agar incubated at 27° C for 72 h.

Hours	Dilutions (Microliter)	Lactic acid bacteria metabolites 1	2	3	4	5	6	7	8	9	10	ANOVA F-Statistic
24	250 µl	0	0	0	0	0	0	0	0	0	0	.000^{ns}
	500 µl	0	0	0	0	0	0	0	0	0	0	.000 ^{ns}
	1000 µl	0	0	0	0	0	0	0	0	0	0	.000 ^{ns}
	Control	0	0	0	0	0	0	0	0	0	0	.000 ^{ns}
48	250 µl	2±0.289^{a}	2±0.289^{a}	2±0.289^{a}	3±0.289^{b}	3±0.289^{b}	2±0.289^{a}	2±0.289^{a}	3±0.289^{b}	2±0.289^{a}	3±0.289^{b}	3.200**
	500 µl	4±0.289^{a}	5±0.289^{b}	4±0.289^{a}	6±0.289^{c}	8±0.289^{e}	6±0.289^{c}	6±0.289^{c}	9±0.289^{f}	8±0.289^{e}	7±0.289^{d}	34.800***
	1000 µl	11±0.289^{a}	12±0.289^{b}	12±0.289^{b}	14±0.289^{d}	18±0.289^{g}	12±0.289^{b}	13±0.289^{c}	17±0.289^{f}	13±0.289^{c}	16±0.289^{e}	68.800***
	Control	0	0	0	0	0	0	0	0	0	0	.000 ^{ns}
72	250 µl	2±0.289^{a}	2±0.289^{a}	2±0.289^{a}	3±0.289^{b}	3±0.289^{b}	2±0.289^{a}	2±0.289^{a}	3±0.289^{b}	3±0.289^{b}	3±0.289^{b}	3.333**
	500 µl	4±0.289^{a}	5±0.289^{b}	4±0.289^{a}	6±0.289^{c}	8±0.289^{e}	6±0.289^{c}	6±0.289^{c}	9±0.289^{f}	8±0.289^{e}	7±0.289^{d}	34.800***
	1000 µl	11±0.289^{a}	12.5±0.289^{b}	13±0.289^{b}	14±0.289^{c}	18±0.289^{e}	13±0.289^{b}	14.5±0.289^{c}	17.5±0.289^{e}	15.5±0.289^{d}	18±0.289^{e}	121.634***
	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 = 0.01. ns = not significant.

Table 6. Effect of LAB metabolites (250 µl, 500 µl and 1000 µl respectively) on Spore germination of *Fusarium verticilliodes* in NBY agar incubated at 27°C for 72 h.

Hours	Dilutions (Microliter)	Lactic acid bacteria metabolites										ANOVA F-Statistic
		1	2	3	4	5	6	7	8	9	10	
24	250 µl	0	0	0	0	0	0	0	0	0	0	0.000^{ns}
	500 µl	0	0	0	0	0	0	0	0	0	0	0.000 ^{ns}
	1000 µl	0	0	0	0	0	0	0	0	0	0	0.000^{ns}
	Control	0	0	0	0	0	0	0	0	0	0	0.000^{ns}
48	250 µl	5±0.289^{c}	5±0.289^{c}	6±0.289^{d}	8±0.289^{e}	6±0.289^{d}	8±0.289^{e}	6±0.289^{d}	2±0.289^{a}	3±0.289^{b}	3±0.289^{b}	50.133***
	500 µl	13±0.289^{e}	12±0.289^{d}	8±0.289^{b}	10±0.289^{c}	10±0.289^{c}	10±0.289^{c}	8±0.289^{b}	6±0.289^{a}	6±0.289^{a}	6±0.289^{a}	75.867***
	1000 µl	21±0.289^{e}	19±0.289^{d}	18±0.289^{c}	18±0.289^{c}	22±0.289^{f}	21±0.289^{e}	21±0.289^{e}	17±0.289^{b}	16±0.289^{a}	19±0.289^{d}	47.467***
	Control	0	0	0	0	0	0	0	0	0	0	0.000^{ns}
72	250 µl	6±0.289^{d}	5±0.289^{c}	6±0.289^{d}	9±0.289^{f}	7±0.289^{e}	9±0.289^{f}	6±0.289^{d}	3±0.289^{a}	3±0.289^{a}	4±0.289^{b}	55.467***
	500 µl	15±0.289^{g}	13±0.289^{e}	10±0.289^{c}	13±0.289^{e}	12±0.289^{d}	14±0.289^{f}	10±0.289^{c}	6±0.289^{a}	6±0.289^{a}	7±0.289^{b}	133.867***
	1000 µl	22±0.289^{e}	20±0.289^{c}	19±0.289^{b}	18±0.289^{a}	23±0.289^{f}	21.5±0.289^{de}	21±0.289^{d}	17.5±0.289^{a}	19±0.289^{b}	20±0.289^{c}	47.215***
	Control	0	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 = 0.01. ns = not significant.

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).

Several works supporting the biopreservation of foods by lactic acid bacterial metabolites have been reported (Singh, 2018). In a similar report by Matei et al. (2016), lactic acid bacterial strains had highly effective antifungal activity against fungal growth and biofilm formation of spoilage fungus *P. expansum.* The metabolites had biopreservative effects on apples.

Conclusion

The application of metabolites from lactic acid bacteria with biopreservative activity in food processing could improve the quality of food and increase its safety by inhibiting food-borne pathogens and spoilage fungi. This study demonstrates that LAB metabolites from fermented cassava can be used as biopreservative agent for food spoilage fungi, as an alternative to chemicals. The production of good amount of bacteriocin has been anticipated to have enormous potential for food applications as biopreservatives and can also be used as food additives during processing to prolong shelf life of such foods.

CONFLICT OF INTEREST

The authors wish to state that there is no conflict of interests regarding the publication of this research work.

REFERENCES

Adss IAAA, Hamza HA, Hafez EE, Heikal HM (2017). Enhancing tomato fruits post-harvest resistance by salicylic acid and hydrogen peroxide elicitors against rot caused by *Alternaria solani.* Journal of Agriculture, Chemistry and Biotechnology 8(1):1-8.

Agrios GN (2005). Plant Pathology, 5th Edition. Elsevier Academic Press USA. pp. 383- 557.

Association of official Analytical Chemists (AOAC) (1990). Methods of Analyses. Association of official Analytical Chemists, Benjamin Franklin Station, Washinton DC.

Avis TJ, Belanger RR (2001). Specificity and mode of action of the antifungal fatty acid cis-9-heptadecenoic acid produced by *Pseudozyma flocculosa*. Applied Environmental Microbiology 67(2):956-960.

Egan K, Field D, Rea MC, Ross RP, Hill C, Cotter PD (2016). Bacteriocins: Novel solutions to age old spore-related problems? Frontiers in Microbiology 7:461.

Hitendra J, Narotham BD, Prasad G, Suvarna H (2016). Role of lactic acid bacteria (LAB) in food preservation. International Journal of Current Microbiology and Applied Sciences 5(8):255-257.

Matei GM, Matei S, Matei A, Cornea CP, Draghici EM, Jerca IO (2016). Bioprotection of fresh food products against

blue mold using lactic acid bacteria with antifungal properties. Romanian Biotechnological Letters 21(1):11201-11208.
National Committee for Clinical Laboratory Standards (NCCLS) (2004). Method for antifungal disk diffusion susceptibility testing of yeasts. Approved guideline M44-A. National Committee for Clinical Laboratory Standards, Wayne, Pa.
Priyanka D, Riddhishah P, Reshma S, Roonal PK, Ashok W (2016). Development of antimicrobial packaging materials for food preservation using bacteriocin from *Lactobacillus casei.* Journal of Biological Research 7(1):6622.
Schillinger U, Lucke FK (2009). Antimicrobial activity of *Lactobacillus sake* isolated from meat. Applied and Environmental Microbiology 55:1901-1906.
Schnurer J, Magnusson J (2005). Antifungal lactic acid bacteria as biopreservatives. Trends in food science and Technology 16(1-3):70-78.
Singh VP (2018). Recent approaches in food biopreservation – a review. Open Veterinary Journal 8(1):104-111.
Simonne A, Ritenour MA, Brecht JK, Sargent SA, Schneider KR (2004). Proper handwashing for produce handlers. Pub. FCS8762-Eng. Department of Family, Youth and Community Sciences, Florida Cooperative Extension Service. University of Florida/IFAS, Gainesville FL 32611. http://edis.ifas.ufl.edu.
Valdés A, Ramos M, Beltrán A, Jiménez A, Garrigós MC (2017). State of the art of antimicrobial edible coatings for food packaging applications. Coatings 7:56.
Ventura M, Zink R (2007). Specific identification and molecular typing analysis of *Lactobacillus johnsonii using* PCR-based methods and pulsed- field gel electrophoresis. FEMS Microbiology 217:141.
Wei W, HaiKuan W (2014).The effect of lactic acid bacteria in food and feed and their impact on food safety. International Journal of Food Engineering 10(2):203-210.