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Inhibitory Activity of Cell Free and Neutralized Cell Free Supernatant of *Lactobacillus* Isolates against Food Spoilage Bacteria

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Abstract

When *Lactobacillus* isolates compete for nutrients, their metabolites which often include active antimicrobials such as lactic acid, acetic acid, hydrogen peroxide, and the peptide bacteriocins can. Inhibitory activity of Cell free supernatant (CFS) and Neutralized Cell free Supernatant (NCFS) of *Lactobacillus* isolates against selected food spoilage bacteria was determined. *Lactobacillus* species used in the study was isolated from cow milk and locust bean samples. Molecular characterization of bacterial isolates from vegetable samples using 16S rDNA gene sequencing_confirmed them as *S. aureus* CIP 9973, *P. carotovorum* Pec1, *E. cloacae* AS10, *K. aerogenes* OFM28, *P. mirabilis* UPMSD3, *E. coli* 2013C-3342, *L. plantarum* NRIC 0383 and *L. plantarum* NCU116. Neutralized cell free supernatant of *L. plantarum* exhibited strong inhibitory activity against *S. aureus* and *E. cloacea*, Similarly NCFS of *L. casei* strongly inhibited the growth of S. *aureus* while no inhibition was observed on the growth of *E. coli, K. aerogenes* and *P. carotovorum.* On the other hand NCFS of *L. acidophilus* was able to inhibit the growth of all six test bacteria with strong inhibition observed on the growth of *K.* The CFS of all three *Lactobacillus* isolates showed varying degrees of inhibition on the isolates from the different vegetable samples with CFS of *L. plantarum* and *L. acidophilus* showing strong zones of inhibition on the growth of *K. aerogenes* and *E. cloacae*, no inhibition zone was

observed on the growth of *P. carotovorum* using CFS of *L. plantarum* and *L. acidophilus*. Cell free supernatant of *L. casei* inhibited the growth of *S. aureus, E. cloacae, K. aerogenes, P. mirabilis* and *E. coli*. No inhibition was observed on the growth of *P. carotovorum* when CFS of *L. casei* was used. The CFS and NCFS of *Lactobacillus* isolates can be utilized as alternate inhibitory agent against food spoilage bacteria.

Keywords: Inhibitory, Activity, Neutralized, Supernatant, *Lactobacillus.*

Introduction

Food spoilage refers to the damage of the original nutritional value, texture, flavour of the food that eventually renders the food harmful and unsuitable to eat (Olubukola *et al*., 2011). One of the major concerns in the food industry is contamination by pathogens, which are frequent causes of food borne diseases. Preservation and safety are presently two major challenges of the food industry because, huge economic losses are sustained yearly due to contamination of food with pathogens while numerous consumers have also been reported to develop adverse sensitivity reactions to chemical based preservatives (Ahmed *et al*., 2017). Microbial food spoilage and food borne diseases over time have become a huge challenge to man; microbes competing with him for available resources in his environment (Ahmed *et al*., 2017). It is well established that food is a valuable source of nutrients for certain microbes (Awojobi *et al.,* 2016), as they grow on the food, they may cause problems such as bad taste, unpleasant smell, and poor appearance (Pei *et al*., 2017). More importantly, the growth of microbes may lead to dangerous levels of toxins in food (Wolska *et al*., 2012). Safe food supply is the best way to reduce incidences of food borne diseases (Shende *et al*., 2016). Despite improvements in food preservation, food borne illnesses caused by bacteria are still a threat to food safety (Patra and Baek, 2017). Food borne diseases are prevalent in the world and are responsible for high morbidity, and mortality (Pei *et al*., 2014). In order to achieve improved food safety against pathogens that cause spoilage of food, the food industry makes use of chemical preservatives or physical treatments (e.g. high temperatures) (Mahdi *et al*., 2011). Despite their potential harm (allergic reaction,

intolerance to some customers, carcinogenic) chemical food preservatives are still in use and it is this background that have given rise to some consumer fears (Tabanelli *et al*., 2014). Increasing demand for safe food has raised the interest in replacing chemical additives by natural products, without injuring the host or the environment (Jang and Gun, 2016). To harmonize consumer demands with the necessary safety standards, traditional means of controlling microbial spoilage and safety hazards in foods are being replaced by combinations of innovative technologies that include biological antimicrobial systems such as lactic acid bacteria (LAB) and their metabolites (Pei *et al*., 2017). Bacteriocinogenic strains of Lactic Acid Bacteria are naturally present in fermented products and contribute not only to the organoleptic characteristics of the products, but play an essential role in natural bio preservation of this products. Lactic acid bacteria (LAB) have been shown to elicit antimicrobial activities and are perceived to be potentially applicable as food bio preservatives (Bhakya *et al*., 2015). Lactic acid bacteria have the potential to inhibit growth of several food borne pathogens and spoilage microorganisms, thereby improving the hygienic quality and shelf life of various food products (Athanasiou *et al*., 2012). Antagonistic properties of Lactic acid bacteria which is allied to their safe history of use in traditional fermented food products makes them very attractive as bio-preservatives (Ansari *et al*., 2012). When LABs compete for nutrients, their metabolites often include active antimicrobials such as lactic acid, acetic acid, hydrogen peroxide, and the peptide bacteriocin (Hwanhlem *et al*., 2013). According to Malheiros *et al*. (2012) lactic acid bacteria have antimicrobial effects on some bacteria pathogens and also play an important role in detoxification of mycotoxins. While scientists are searching for efficient strategies to overcome limitations in the use of bacteriocins, it is important to explore the use of other bacteriocins in as antibacterial agents that could possibly be applied in the food industry and have the potential to revolutionize the global food system. This study aimed at determining the Antibacterial activity of Cell free and Neutralized cell free supernant on selected Food spoilage bacteria

Methodology

Sampling technique used in the study

Samples were collected from different locations, points and times with sterile materials (calibrated plastic containers, sterile polythene zip bags and universal bottles) after which they were transferred to the Microbiology Laboratory of the Federal University of Agriculture Makurdi for further analysis.

Bacterial Isolates

Bacterial isolates used as test organisms were obtained from the Microbiology department of the Federal University of Technology Makurdi and molecularly identified as *Staphylococcus aureus CIP* 9973, *Pectobacterium carotovorum* subsp. *carotovorum* Pec 1, *Enterobacter cloacae* AS10, *Klebsiella aerogenes* OFM28, *Escherichia coli* 2013C-3342 and *Proteus mirabilis* UPMSD3 were used as indicator microorganisms for the quantification of bacteriocin-like inhibitory substance (BLIS) produced by *Lactobacillus plantarum.* NRIC 0383. All strains were maintained in glycerol (20% v/v) at -80°C as stock culture.

Processing of samples

Processing of locust bean

Locust bean sample (300 g) was weighed using a weighing scale, and crushed into tiny bits using a mortar and pestle pre sterilized using 70% alcohol. The different homogenates were collected in labeled sterile tubes and stored in sterile containers at 4ºC in the refrigerator (Haier Thermocool, China) for further use.

Pasteurization of cow milk

Raw cow milk was poured into a sterile stainless steel pot and heat slowly to 72 ºC with stirring. The temperature was held at 72ºC for exactly 15 seconds after which the pot of milk was removed from the heat and placed in a large bowl filled with ice water (Hata *et al*., 2010). Pasteurized raw cow milk sample was then transferred into sterile 500 ml beaker, covered with aluminum foil and left on the bench to ferment.

Isolation of *Lactobacillus* species from cow milk and locust beans samples

Processed locust beans and cow milk samples were serially diluted, by transferring 1 g/1 ml of each sample separately into 9 ml of sterile water to make a stock mixture. Homogenized mixture (1 ml) was then taken into appropriately labelled test tube to make 10^{-1} of the mixture. The serial dilution was continued until 10^{-8} was obtained. An aliquot (0.1 ml) of the respective dilutions was spread over MRS (de Man, Rogosa, Sharpe) agar plates. The MRS agar plates were then incubated anaerobically in an anaerobic jar (Microbiology AnaerotestX Merck, Darmstadt, Germany) at 37 ± 2°C for 48 hours. Growth on MRS plates were then observed after 48 hours.

Purification of bacteria isolates

Colonies from cow milk and locust beans samples cultured on MRS agar plates were sub cultured twice on MRS agar and incubated in an incubator (Swiss *model* NU-5700, UK) at 37 ± 2°C for 48 hours to get pure colonies. Purified isolates from vegetable, cow milk and locust bean samples were maintained at 4°C (Haier Thermocool, China) in MRS and nutrient broth medium respectively for further characterization and identification.

Identification and Characterization of LAB Isolates from cow milk and locust beans

Lactic acid Bacteria isolates were identified macroscopically, microscopically (based on colonial and cellular morphology), and by various biochemical means. Their identities were further confirmed using 16S rDNA gene identification.

Biochemical tests for the identification of isolates

Biochemical tests for the identification of the different isolates were performed according to Bergey's manual of systematic bacteriology (Whitman *et al*., 2012).

Gram staining

Gram stain was carried out by placing a drop of water on a clean glass slide, part of a single colony taken aseptically from freshly grown culture was mixed with the water to make a smear. It was passed through Bunsen flame intermittently to fix the smear to the glass slide. The smear was flooded with

crystal violet for one minute and rinsed under running tap water. It was further flooded with Gram iodine for 30 seconds and rinsed gently with tap water. The smear was then decolourized by adding 70% (v/v) ethanol to the cells and rinsed off with tap water immediately until no colour effluent was seen. A counter stain, safranin was added to the smear for 1 minute, rinsed with water, drained and air dried. Under a compound light microscope (Bausch and Lomb Dynazoom, SMZ1500) using oil immersion objective lens (x 100) the smear was observed for cell colour and shape. The smear was also observed for the presence of more than one type of cell to check the purity. Gram positive cells showed a purple colouration while the red or pink coloured cells indicated Gram negative cells (Fawole and Oso, 2001).

Sugar fermentation test for all isolates

Sugar fermentation patterns were assessed according to the method of Whitman *et al*. (2012). Ten percent solution of each sugar to be used (Glucose, arabinose, mannitol, maltose, sucrose, galactose, raffinose and lactose) was added to peptone water using phenol red as the indicator and dispensed into test tubes containing inverted Durham's tubes. These were then autoclaved (Hydrolab, DWK Life Sciences, Duran) at 121 ^{o}C for 5 min and inoculated with each isolate. One test tube of each medium was left un-inoculated serving as control. The tubes were then incubated at 37 ± 2°C for 24 hours; a colour change indicated acid production in all sugars used. Airspace in the Durham's tube showed gas production.

Inoculum preparation and standardization

Inoculum was prepared by aseptically inoculating a colony picked from each of the culture streaked plates into sterile 9 ml De Mann Rogosa Sharpe Agar (MRS) and NB broth in sterile capped bottles using sterile inoculating loop. The broth was then incubated with shaking at 10,000 rpm using an orbital shaker (IKA™ 10316411) at 37°C until the visible turbidity was equal to or greater than that of 0.5 McFarland standard (NCCLS, 1999).

Determination of the antimicrobial spectrum of LAB isolates against test organisms

Antibacterial spectrum of purified isolates from fermented cow milk and locust bean against the test bacteria (*Escherichia coli*, *Pectobacterium carotovorum*,

Enterobacter cloacae, *Klebsiella aerogenes*, *Staphylococcus aureus* and *Proteus mirabilis*) was confirmed using the Agar well diffusion assay.

Agar well diffusion assay

Antimicrobial activity of isolates from fermented cow milk and locust bean samples using the agar diffusion method was carried out according using the method of Ogunbanwo *et al*. (2003). Brain heart infusion soft agar was seeded separately with 2 ml (10^8 CFU/ml) of the various test bacteria, mixed and poured into sterile Petri dishes. After setting, agar wells of 8 mm in diameter were punched into the agar plates using sterilized well cutter. Fifty microlitre (50 µl) of each of the *Lactobacillus* isolates earlier suspended in 20 ml of MRS Broth was carefully pipetted into each well and the plates were incubated for 24 hours at the optimum temperature of each test bacteria. Uninoculated MRS broth served as control. The incubated plates were examined for zones of clearance around the individual wells. The diameter of the transparent zones were measured using a ruler (mm). Tests was carried out in triplicates. Isolates that had greater zones of inhibition were further screened for bacteriocin production.

Screening of bacteriocin-producing isolates

Preparation of cell-free supernatant (CFS) and neutralized cell free supernatant (NCFS)

Isolates from cow milk and locust beans that showed significant zones of inhibition (*Lactobacillus plantarum, Lactobacillus acidophilus* and *Lactobacillus casei*) against the test bacteria were incubated in 20 ml of MRS broth at 37 ± 2ºC for 24 hours in an anaerobic jar (Microbiology Anaerotest X Merck, Darmstadt, Germany). Cell-free supernatant (CFS) was then obtained by centrifugation at 10,000 g for 10 minutes (Sorvall RC6 PLUS, Thermo-electron Corporation, Asheville, NC, USA) to separate bacterial cells from supernatant. The supernatant was filtered through a sterile 0.22 µm syringe filter (Chromatographic Specialties Incorporated, Canada). Neutralized cell free supernatant (NCFS) was prepared by adjusting the pH of CFS to 7.0 with 1 ml of 1N NaOH to exclude the antimicrobial effects of organic acids. Inhibitory activity due to hydrogen peroxide (H_2O_2) was eliminated by the addition of 1 ml of catalase. Samples were then heat to 100ºC using a water bath for 10 minutes to inhibit enzyme activity. The CFS and NCFS were stored at 20ºC in

3 ml of 0.05 M phosphate buffer until needed for screening the sensitivity of isolated test bacteria by the agar well diffusion assay (Noraphat *et al.,* 2017).

Determination of the antimicrobial activity of CFS AND NCFS of selected *Lactobacillus* species against test bacteria

Antimicrobial activity of CFS and NCFS of *Lactobacillus plantarum, Lactobacillus acidophilus* and *Lactobacillus casei* was determined using the method of Noraphat *et al.* (2017). This was done by inoculating one ml of each test bacteria (*Escherichia coli*, *Staphylococcus aureus*, *Pectobacterium carotovorum*, *Enterobacter cloacae*, *Klebsiella aerogenes* and *Proteus mirabilis*) previously adjusted to 1.5×10^8 CFU/ml in 20 ml of semisolid brain heart infusion agar (BHI) medium maintained at 50 °C and then poured into a Petri dish. After solidification, two wells (8 mm diameter) were bored using a well borer. Fifty microliter of cell-free supernatant (CFS) of *L. plantarum, L. acidophilus* and *L. casei* were added to each well. Neutralized CFS (NCFS) of *L. plantarum, L. acidophilus* and *L. casei* which were earlier treated with 1 ml of catalase (Sigma-Aldrich Corporation, USA) at 25 °C for 30 mins to eliminate the possible inhibitory action of H_2O_2 that was previously adjusted to pH 7.0 with 1 m of NaOH in order to rule out possible inhibition effects due to organic acids, were then placed in the second well. After an incubation period of 37 ± 2 ^oC in the incubator (Swiss *model* NU-5700, UK) for 24 h, incubated plates were examined for zone of clearance around the individual wells. If inhibition zones were found in the second well, the isolates were considered to be able to produce inhibitory substances (IBS).

Data Analysis

Statistical analysis of numerical data obtained throughout the bench work of this research were carried out with the aid of Microsoft Excel and Statistical Package for Social Sciences (SPSS version 22). Analysis of variance (ANOVA) of data obtained were used to detect differences in means among treatments. Tukey honest significant difference (HSD) test was used to determine the difference in mean between pairs of treatment. A 5% (0.05) level of significance was used for all statistical tests conducted.

RESULTS

Occurrence of LAB isolates from locust beans and fermented cow milk samples are outlined in Table 1. *Lactobacillus acidophilus* and *L. casei* were isolated from locust beans samples, while *L. plantarum* in addition to *Lactobacillus acidophilus* and *L. casei* were isolated from fermented cow milk sample (Table 1).

Table 1: Occurrence of *Lactobacillus* Isolates in Locust Beans and Cow Milk Samples

Point of collection	Sample	*Lactobacillus* occurrence
Vendor A	Locust bean	*Lactobacillus acidophilus*
Vendor B	Locust bean	*Lactobacillus acidophilus, Lactobacillus casei*
Vendor C	Locust bean	*Lactobacillus casei, Lactobacillus acidophilus*
Vendor A	Fermented milk	*Lactobacillus acidophilus, Lactobacillus casei, L. plantarum*
Vendor B	Fermented milk	*Lactobacillus plantarum, Lactobacillus acidophilus*
Vendor C	Fermented milk	*Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus plantarum*

Growth was prominent on Man Ragosa Sharpe agar plates containing samples from locust bean and cow milk after 48 hours. A total of 15 different colonies were observed from locust bean and cow milk samples (8 from 'cow milk' and 7 from 'locust bean'). The colonies were screened for their antibacterial activity against the test bacteria (*E.coli*, *P. carotovorum*, *E. cloacae, K. aerogenes*, *S. aureus* and *P. mirabilis*) isolated from the vegetable samples, isolates which showed reasonable inhibitory action against at least one of the test bacteria were selected. Three isolates were found to demonstrate antibacterial activity against the test bacteria. The isolates produced small, irregular, round, shiny white brownish colored colonies on MRS agar which were morphologically similar to *Lactobacillus* specie. Microscopic features of isolates from

MRS agar plates showed gram positive, short, medium rod shaped non-spore forming bacteria. The isolates were found to be catalase and oxidase negative and in IMVC (indole, methyl-red, vogues proskauar, citrate) they were also proved negative but positive for methyl red and were able to reduce nitrate and ferment different carbohydrates (Glucose, arabinose, mannitol, maltose, sucrose, galactose, Raffinose and Lactose) to varying levels indicating that they are able to grow in a variety of habitats while utilizing different types of carbohydrates. When checked for gas production, all of them gave negative result (Table 2).

Table 2: Biochemical Characteristics of *Lactobacillus* isolates with Antimicrobial activity against the Test Bacteria

Isolate	Morph	Gram	Motilit	Spore	Oxidas	Catalas	Indole	H_2S	Glucos	Arabin	Mannit	Maltos	Sucros	Galact	Nitrate	Raffin	Lactos	Gas	Identified
IS1	Rod	+	-	-	+	-	-	+	+	-	-	+	+	-	-	+	+	+	*Lactobacillus acidophilus*
IS3	Rod	+	-	-	+	-	-	+	+	+	+	+	+	-	-	+	+	+	*Lactobacillus plantarum*
IS3	Rod	+	-	-	+	-	-	+	+	+	+	+	+	-	-	+	+	+	*Lactobacillus casei*

Antimicrobial activity of the different LAB (*Lactobacillus acidophilus, L. casei, L. plantarum*) isolates from fermented cow milk (FCM) and locust beans (LB) against the different test bacteria using the agar well diffusion showed that all three isolates were able to inhibit the growth of the test bacteria to varying levels. *Lactobacillus plantarum* showed maximum inhibitory activity ranging from 26.00 ± 1.15 mm to 20.10 ± 0.84 mm for agar diffusion, with highest

activity against *S. aureus* (26.00 ± 1.15 mm), *P. carotovorum* (23.21± 0.62 mm) and *E. cloacae* (22.00 ± 0.68 mm). Maximum inhibitory activities of *L. acidophilus* on the growth of *E. cloacea* and *P. carotovorum* were 21.30 ± 0.03 mm and 20.50 ±1.07 mm. *Lactobacillus casei* recorded the least inhibitory activity of 9.24 ± 0.84 mm on *Enterobacter cloacae* AS10 using the **a**gar well diffusion assay (AGWD) (Table 3).

Table 3: **Antimicrobial Activity of *Lactobacillus* Isolates against the Test Bacteria using the Agar Diffusion Assay**

	Diameter of inhibition zone (mm) for test bacteria						
LAB	***E. cloacae***	***E.coli***	***K. aerogenes***	***P. carotov***	***P. mirabilis***	***S. aureus***	**Overall mean**
L. acidophilus	21.30 ± .03^{a}	14.20 ± 1.33^{c}	18.30 ± 0.68^{a}	20.50 ±1.07^{b}	16.22 ± .62^{b}	18.42 ± 1.25^{b}	18.16 ± 2.6^{b}
L. casei	9.24 ± .84^{b}	12.50 ± 1.25^{b}	11.00 ± 1.33^{b}	16.80 ±2.00^{c}	14.20 ± .51^{c}	16.39 ± .51^{c}	13.36 ± 2.9^{c}
L. plantarum	22.00 ± .68^{a}	20.10 ± 0.84^{a}	19.00 ± 1.25^{a}	23.21 ±0.62^{a}	21.00 ± .48^{a}	26.00 ±1.15^{a}	21.89 ± 2.4^{a}

Values are means of three replicates ± standard deviation. Means within a vertical column with the same superscript are not significantly different according to Tukey HSD post-hoc test at 5% level of significance.
Over all mean: Over all mean activity of LAB across test bacteria
LAB = Lactic acid bacteria*; E. cloacae = Enterobacter cloacae*; *E. coli = Escherichia coli*

K. aerogenes = *Klebsiella aerogenes*; *P. mirabilis* = *Proteus mirabilis*; *P. carotovorum* = *Pectobacterium carotovorum ; S. aureus* = *Staphylococcus aureus*

Cell Free supernatant (CFS) and Neutralized cell free supernatant (NCFS) of the different *Lactobacillus* isolates exhibited varying inhibitory activities using the agar well diffusion assay against the different bacteria isolates from the selected vegetables. Neutralized cell free supernatant of *L. plantarum* exhibited strong inhibitory activity against *S. aureus* and *E. cloacea*, while a mild inhibition was observed on the growth of *P. carotovorum*, *K. aerogenes*, *P. mirabalis*, *E. cloacae* and *E.coli* using NCFS of *L. plantarum* and *L. casei.* Similarly NCFS of *L. casei* strongly inhibited the growth of S. *aureus* while no inhibition was observed on the growth of *E. coli, K. aerogenes* and *P. carotovorum.* On the other hand NCFS of *L. acidophilus* was able to inhibit the growth of all six test bacteria with strong inhibition observed on the growth of *K. aerogenes* (Table 4). The CFS of all three *Lactobacillus* isolates showed varying degrees of inhibition on the isolates from the different vegetable samples with CFS of *L. plantarum* and *L. acidophilus* showing strong zones of inhibition on the growth of *K. aerogenes* and *E. cloacae*, no inhibition zone was observed on the growth of *P. carotovorum* using CFS of *L. plantarum* and *L. acidophilus*. Cell free supernatant of *L. casei* inhibited the growth of *S. aureus, E. cloacae, K. aerogenes, P. mirabilis* and *E. coli*. No inhibition was observed on the growth of *P. carotovorum* when CFS of *L. casei* was used (Table 4). Control which was MRS broth showed no inhibition on the growth of isolates from the vegetable sample (Table 4).

Table 4: **Antimicrobial Spectrum of CFS and NCFS of LAB against the Test Bacteria**

LAB isolates	Individual treatment	Test bacteria					
		S. aureus	*P.carotovorum*	*E. cloacae*	*K.aerogenes*	*P.mirabilis*	*E. coli*
L. plantarum	CFS	+	-	++	+	+	-

	NCFS	++	+	++	+	+	+
L. casei	CFS	+	-	+	+	+	+
	NCFS	++	-	+	-	+	-
L.acidophilus	CFS	+	-	+	++	+	+
	NCFS	+	+	+	++	+	+
Control	CFS	-	-	-	-	-	-
	NCFS	-	-	-	-	-	-

CFS: Cell free supernatant; Control: MRS broth ; NCFS: Neutralized cell free supernatant (Adjusted to pH 7 and 1 ml of catalase added); - : no inhibition; +: diameter of inhibitory zone within 5-10 mm (mild); ++: diameter of inhibitory zone within 10-15 mm (Strong).

DISCUSSION

The presence of lactic acid bacteria (LAB) in cow milk and locust been used in this study was an evidence of LAB as a normal microflora of fermented foods. It also shows that traditional fermented food products can potentially be good sources of probiotic organisms, producing antimicrobial substances such as organic acids, diacetyl, hydrogen peroxide and bacteriocins which are suspected to be associated with the preservation of many fermented food condiments in Nigeria. Njoku and Okemadu (1998) also reported diverse groups of microorganisms including *Bacillus, Micrococcus, Leuconostoc, Staphylococcus, Enterobacteriaceae* and lactic acid bacteria (LAB) that play active roles in the process of fermentation. Bacteriocins produced by different strains of *L. plantarum* isolated from food products have also been described by Ogunbawo *et al*. (2003). Tajabadi *et al*. (2011); Ravi *et al*. (2012); Zhao *et al*. (2016) and Balogun *et al*. (2017) also isolated LAB from yogurt, cheese, fermented milk and dough. These indicated that fermented foods represent an abundant resource of such potentially useful bacteria. Lucera and Del (2012) reported that most bacteriocin-producing strains, particularly those producing class IIa bacteriocins, have been isolated from meat products.
Summarized results of biochemical tests for bacteria isolates from the vegetable samples were in agreement with those reported by Khairuzzaman *et al*. (2014).

Biochemical tests result for *Lactobacillus i*solates which showed that they were catalase and oxidase negative and in IMViC (Indole, Methyl-red, Voges proskauar, Citrate utilization) tests were also negative confirmed that the isolates were *Lactobacillus* species. This is in agreement with the findings of Dhanasekaran *et al*. (2010) and Saranya and Hemashenpagam (2013) who both observed same result for *Lactobacillus* species isolated from Yoghurt. Of the 15 isolates from cow milk and locust bean samples only three (3) exhibited reasonable inhibitory activity against the test bacteria, this could be due to factors such as the incubation conditions and target organisms. Ravi *et al*. (2012) and Saranya and Hemashenpagam (2013) also isolated low numbers of *Lactobacillus* strains from fermented foods. According to Noraphat *et al*. (2017), culture medium, incubation conditions, target microorganisms as well as the sensitivity of methods used in determining the antimicrobial activity are important factors. The isolates were able to ferment 11 different carbohydrates, i.e., glucose, sucrose, fructose, lactose, xylose, ribose, galactose, maltose, mannitol, rhamnose and dextrose indicating that they were able to grow in a variety of habitats utilizing different types of carbohydrates.

Antibacterial activities of *L. plantarum, L. acidophilus* and *L. casei* observed against the test bacteria which consisted of gram negative and positive isolates (*S. aureus, P. carotovorum, E. cloacae, K. aerogenes, P. mirabilis* and *E. coli*) were largely, but not totally, due to its ability in its undissociated form to penetrate the cytoplasmic membrane, resulting in reduced intracellular pH and disruption of the transmembrane proton motive force (Zacharof and Lovitt, 2012). Gram-positive bacterium such as *S. aureus* has a thick mesh-like cell wall made of peptidoglycan (90% of the cell wall), while Gram-negative bacterium (*P. carotovorum, E. cloacae, K. aerogenes, P. mirabilis* and *E. coli* has a thinner layer (10% of the cell wall). Gram-negative bacteria also have an additional outer membrane, which contains lipids and is separated from the cell wall by the periplasmic space (Mamlouk *et al*., 2012). As reviewed by Nikaido (1996), the relative efficacy of lactic acid bacteria against gram-negative bacteria was not unexpected considering that as a small water-soluble molecule lactic acid gains access to the periplasm through the water-filled porin proteins of the outer membrane (OM), however the OM has been known to function as an efficient permeability barrier that is able to exclude macromolecules (such as bacteriocins or enzymes) and hydrophobic substances (i.e., hydrophobic antibiotics). The permeability barrier property of the OM is largely due to the

presence of a specific lipopolysaccharide (LPS) layer on the membrane surface (Helander *et al*., 1996). According to Zhao *et al*. (2016), when LABs compete for nutrients, their metabolites often include active antimicrobials such as lactic acid, acetic acid, hydrogen peroxide, and the peptide bacteriocins. Sivaramasamy *et al*. (2014) also reported that LAB have the capability to produce various compounds, such as acetic acid, hydrogen peroxide, ethanol, diacetyl and bacteriocins that contribute to the inhibitory effects on pathogenic microorganisms. In agreement Yang *et al*. (2012) reported that some bacteriocins are able to cause lethal injury to *Escherichia coli*, similar properties have also been ascribed to acetic acid and indirect evidence inferred that such injury involved disruption of the LPS layer.
Inhibitory activity of cell free supernatant (CFS) and neutralized cell free supernatant (NCFS) of *L. plantarum, L. acidophilus and L. casei* against the test bacteria as determined by the well diffusion assay showed that the activity of NCFS of lactic acid bacteria was not associated with the production of organic acids and hydrogen peroxide (H_2O_2) as their effects were earlier eliminated using catalase and by adjusting the CFS to pH 7. The wider spectrum of activity of NCFS of *L. plantarum* and *L. acidophilus* against the test bacteria as compared to that of *L. casei* suggested that the NCFS of *L. plantarum* and *L. acidophilus* produced more bacteriocin when compared to *L. casei.* It also showed that lactic acid and hydrogen peroxide (H_2O_2) was largely, but not totally responsible for antimicrobial LAB activity. The antimicrobial effect of CFS of *L. plantarum, L. acidophilus and L.casei* may also have resulted from the oxidation of sulfhydryl groups that could have caused denaturing of a number of enzymes, and possibly the peroxidation of membrane lipids, thus increasing membrane permeability that adversely affected the target bacteria (Hwanhlem *et al*., 2013). This is in agreement with the results of Ogunbawo *et al*. (2003) and Balogun *et al*. (2017) who observed that NCFS of *L. plantarum* had a wider inhibitory activity as compared to *L. casei*. Similarly, results obtained in this study is also in agreement with that of Lucera and Del (2012) who highlighted increased interest in the use of bacteriocin producing LAB, or their more or less purified form in food preservation. The inhibitory activity of the cell free supernant of *L. plantarum, L. acidophilus and L .casei* against the test bacteria buttressed the fact that the antibacterial effect of organic acids lies in the reduction of pH, as well as the generation of undissociated forms of the molecules. According to Celia *et al*. (2018) undissociated lactic acid acts by

inhibiting the electrochemical proton gradient or by altering the cell membrane permeability, which results in disruption of substrate transport systems in the organism of interest. Increased inhibitory activity of CFS could also be attributed to the presence of hydrogen peroxide, which according to Ansari *et al*. (2012) is one of the antimicrobial compounds produced by LAB in the presence of oxygen which results from the action of flavoprotein oxidases or nicotinamide adenine dinucleotide (NADH) peroxidase.

CONCLUSION

Locust bean and cow milk samples used in the study were good sources of Lactic acid bacteria (LAB). Characterization of isolates of LAB revealed the occurrence of *Lactobacillus plantarum, L. acidophilus* and *L. casei.* Antibacterial activities of *L. plantarum, L. acidophilus* and *L. casei* observed against the test bacteria which consisted of gram negative and positive isolates (*S. aureus, P. carotovorum, E. cloacae, K. aerogenes, P. mirabilis* and *E. coli*) were largely, but not totally, due to its ability in its undissociated form to penetrate the cytoplasmic membrane, resulting in reduced intracellular pH and disruption of the transmembrane proton motive force.

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