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Full Length Research Paper

Lactic acid bacteria from traditionally processed corn beer and palm wine against selected food-borne pathogens isolated in south west region of Cameroon

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The present study was undertaken to assess the inhibitory potential of lactic acid bacteria isolated from traditionally processed corn-beer and palm-wine on *Escherichia coli, Salmonella typhi* and *Staphylococcus aureus.* Lactic acid bacteria were isolated on MRS agar using pour plate method. The catalase negative and Gram positive isolates were selected as presumptive lactic acid bacteria and were biochemically characterized using the API 50 CHL BioMerieux kit to identify them at species level. The LAB isolates were then assessed for antimicrobial activity potentials against food-borne pathogens. Thirteen LAB isolates which constituted nine different species namely: *Lactobacillus fermentum*, *Lactobacillus plantarum, Lactobacillus brevis, Lactobacillus acidophilus, Lactobacillus bulgaricus, Leuconostoc mesenteroides, Leuconostoc carnosum, Pedioccocus acidilactici* and *Lactobacillus pentosus* were isolated from these two beverages. The entire LAB isolates demonstrated great potentials to inhibit the test pathogens. *P. acidilactici* from corn-beer exhibited the overall highest inhibitory activity with zones of inhibition of 19, 20 and 16 mm on *E. coli, S. typhi* and *S. aureus,* respectively; while the isolate from palm-wine, *L. pentosus* exerted the highest antimicrobial action on the test pathogens. It was observed that most of the LAB isolates inhibited the indicator pathogens mainly by bacteriocin production. *S. typhi* was the most susceptible food-borne bacterial pathogen to the inhibitory activity of the LAB isolates, followed by *E. coli*.

Key words: Lactic acid bacteria, food-borne pathogens, antimicrobial activity, probiotics, bacteriocins.

INTRODUCTION

The occurrence of foodborne microbial pathogens and the increased foodborne toxi-infections remain a critical issue in many countries around the world (Newell et al., 2010). The development of foodborne diseases is a

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major health problem in underdeveloped and developing countries. Sub-Saharan Africa is one of the most affected regions (Quilan, 2013). This is associated with poverty and poor hygiene conditions. Salmonellosis, occupy an important place. A recent survey carried out by Nguendo (2014) reveals that 70% of food borne illness cases in Cameroon is associated with the consumption of street foods sold in major cities such as Douala and Yaoundé. Methods used for the treatment of these diseases involve mainly the use of antibiotics. However, the prolonged use of antibiotics in these regions has been indexed as the main cause of the occurrence of bacterial strains resistant to antibiotics. The emergence of antibiotic resistant strains is a major public health problem (Tatsinkou et al., 2016). The exploitation of the inhibitory activity of the lactic bacteria appeared in recent years as an alternative treatment and even prevention of some foodborne toxi-infection (Mezaini et al., 2009; Obi et al., 2015; Khalid, 2011). Indeed the lactic acid bacteria are known to be non-pathogenic and Generally Regarded as Safe, and are increasingly used because of their probiotic properties (Zacharof and Lovitt, 2012; Mariam et al., 2014).

Probiotics are microbial strains and administration in adequate amount is beneficial in humans or animals. Many recent studies have demonstrated the potential of LAB to inhibit the growth of food borne pathogens by various mechanisms, including the production of antimicrobial proteins called bacteriocins (O'Shea et al., 2013).

The traditional processed corn and palm wines are widely consumed in Africa and particularly in Cameroon. They have recently been described as potential niches of lactic acid bacteria. Which are partly involved in the process of fermentation and the flavor of these native drinks. To the authors' knowledge, very few studies have been conducted on the probiotic potential of lactic acid bacteria strains isolated from traditional processed corn beer and palm wine; hence, the need to conduct a study on the probiotic potential of these strains, mainly the inhibitory activity against food pathogens.

MATERIALS AND METHODS

Study site and sample collection

This study was conducted in Fako Division of the South West Region of Cameroon. The specimens were obtained from Buea, Limbe, Tiko and Muyuka, these being the four major towns in Fako division, South west Region of Cameroon. The samples were processed in the laboratory of the School of Assistant Laboratory Technicians of Limbe and the Biotechnology unit of the University of Buea.

Three samples of each of the two specimens (corn-beer and Palm wine) were collected from the four towns in Fako Division to give a sample size of 24. About 50 ml of each fresh sample was poured in sterile wide-mouth collection cups and labelled and the temperature was taken at the spot. Samples obtained in towns further from Limbe were transported in cool packs. Upon arrival at the laboratory, the pH of each sample was measured by using calibrated Universal Litmus pH test strips (LabRat Supplies). Then each sample was separated in two cups. All the samples were continuously labelled as they were being separated. The pH was measured so as to ascertain whether the antimicrobial potentials of the lactic acid bacteria had any relationship with acidity of the samples.

Media preparation and sample processing

All media were prepared following the manufacturer's instruction. The media used were de Man Rogosa and Sharpe (MRS) (Oxoid) agar for isolation of LAB, MRS Broth (Oxoid) for the production of antimicrobials, Nutrient Agar (Liofilchem s.r.i Bacteriology products) for total microbial count, and Muller Hinton Agar (Oxoid) for antimicrobial sensitivity testing. Aseptic techniques were observed throughout the media preparation process. A 1:10 dilution of each sample was made prior to culturing. This was done by diluting 1 ml of the sample within 9 ml of physiological saline (0.85% NaCl). Further, ten-fold serial dilutions ranging from 10^{-1} to 10^{-5} were prepared. The 10^{-5} diluted samples were used for culture on MRS Agar and Nutrient agar.

Isolation and phenotypic identification of LAB

Samples for the isolation of lactic acid bacteria were cultured on MRS Agar. The pour plate method was employed; about 1 ml of each of the 10^{-5} diluted sample was pipette into separate sterile plates and about 15 ml of the prepared molten MRS agar was poured on it. The plates were then gently rotated clockwise and anti-clockwise so as to allow for a homogeneous distribution of the agar and the diluted sample. The agar was allowed to solidify, then inverted and incubated at 30°C for 48 h.

At the end of the incubation period, the MRS plates were observed for colony formation. Colonies which were different from each other in their morphology and phenotypic appearance were picked up using a sterile inoculating loop and were purified on MRS Agar by re-streaking on plates until only a single type of colony was present. The different pure cultures so obtained were characterized for their colony morphology and subjected to Gram staining and catalase test. Colonies found Gram positive, non-motile, rod shaped bacteria that demonstrated a catalase negative result were selected as presumptive lactic acid bacteria. They were then preserved on MRS Agar slants and store at 4°C for further investigations.

The identification of lactic acid bacteria at species level was done by biochemical characterization using the API 50CH kit (BioMerieux, France). The API 50 CH is a standardized system that associates the fermentation of 50 carbohydrates to bacteria species. It is used for the identification of *Lactobacillus* and related genera.

A positive test corresponds to the acidification revealed by the bromocresol purple indicator contained in the medium changing to yellow. For the esculin test (tube no. 25), a change in colour from purple to black was interpreted as positive (+). The biochemical profiles obtained for the LAB strains was analyzed using the API identification software database (API LAB PLUS), Version 5.

Determination of antimicrobial activity

Preparation of the sample filtrate

Each presumed LAB isolate was inoculated from slants into 5 ml of MRS broth and incubated at 37°C for 24 h. The culture broth of each isolate was then centrifuged at 6,000 rpm for 10 min. The cell free supernatant (CFS) was then collected and passed through a 0.2µm sterile syringe filter and stored at -20°C prior to use. The CFS was thus used for susceptibility testing, determination of

minimum inhibition concentration (MIC) and determination of the antimicrobial substances.

Sources of food-borne pathogens

The food borne bacteria pathogens that were used for this study were provided by the Laboratory of Microbiology of the Buea Regional Hospital Annex and Limbe Regional hospitals. They were isolated from stools of patients with symptoms of food borne diseases and characterized and identified by API 20 A, 20 E, 20 Staph Kits (BioMerieux, France). We were given pure cultures that were store on slant and preserved at 4°C.

Susceptibility testing by the agar well diffusion method

The Agar well diffusion methods suggested by Tagg and McGiven (1971), was modified and used to test for the antimicrobial activity of the presumed LAB isolates against selected food borne pathogens. The pure cultures of the selected foodborne pathogens were inoculated from slants to about 5 ml Muller Hinton broths and incubated for 24 h at 30°C. About 1% of the 24 h culture of the pathogen was suspended in 9 ml of normal saline and adjusted to 0.5 McFarland standards. A lawn of the indicator strains were then made by spreading the cell suspension over the surface of Muller Hinton agar plates with a sterile cotton swab. The plates were allowed to dry and a sterile cork borer of diameter 5 mm was used to bore uniform wells in the agar. Each well was then filled with 100 µl of the different concentrations of the cell-free supernatant (LAB culture filtrate). After incubation at 37°C for 48 h, the plates were observed for a zone of inhibition around the well. The antimicrobial activity was expressed as the diameter of the inhibition zones around the wells. Results were considered positive if the diameter of the zones of inhibition were greater than or equal to 7 mm. The negative control used was 100 µL of distilled water while the positive control used were 100 µl of diluted Ciprofloxacin and Azithromycin.

Determination of minimum inhibition concentration

The MIC was performed using the broth dilution method, by following the modified version of the procedure described in the BSAC Guide to Sensitivity Testing (1991). In this method, 4 to 5 isolated colonies of the test pathogens were obtained and cultured overnight at 37°C on Muller Hinton agar. The cultures were standardized using standard microbiological techniques to have a concentration of very near 1 million cells per milliliter (10^6 cfu/mL). After overnight incubation of the test pathogen, the CFS (antimicrobial substance) of LAB isolates were then diluted using Muller Hinton Broth, beginning with 1/2 dilution up to 1/12 serial dilutions. The different test pathogens were then inoculated into each of the diluted tests tubes to a final density of 5×10^5 cfu/ml. The tubes were then incubated for 18 h at 37°C. After the incubation time, the tubes were inspected for growth of the test pathogens. Meanwhile, aliquots of growth control (with no antimicrobial substance) were plated to verify cfu/ml counts of viable bacteria. These were equally incubated under the same conditions and colonies were counted. The MIC was recorded as the lowest concentration of the antimicrobial substance that prevents the appearance of visible turbidity.

Determination of inhibitory substance

The Cell free supernatant (CFS) of each LAB Isolate was prepared as stated above. Each sample filtrate was then separated into 3 different sterile test tubes for the determination of antimicrobial substance due to bacteriocin, acid and hydrogen peroxide, respectively. The process was as follow:

1. The CFS in the first tube was used to determine whether the antimicrobial activity is due to bacteriocin. The CFS in this tube was thus adjusted to pH 7.0 by the addition of sterile 1 N sodium hydroxide (NaOH), to eliminate any effect of acidity; while inhibitory activity due to hydrogen peroxide was eliminated by the addition of a catalase enzyme (5 mg/dl), Amplex Red Catalase Assay Kit (A22180). The filtrate was then used for susceptibility studies.
2. The CFS in the second tube was used to determine whether the inhibitory substance is an acid. Thus, catalase enzyme (5 mg/dl) was added to this tube to eliminate any inhibitory activity due to hydrogen peroxide. The tube was then heated in boiling water (100°C) to denature proteins and thus eliminate any inhibitory activity due to the presence bacteriocin in the CFS.
3. The CFS in the third tube was used to determine whether the inhibitory substance is hydrogen peroxide (H_2O_2). Here, the CFS was adjusted to pH 7.0 by the addition of sterile 1 N NaOH to eliminate inhibitory effect due to acid.

The catalase enzyme (5 mg/dl) was added to this tube to eliminate any inhibitory activity due to hydrogen peroxide. The pure cultures of the selected food borne pathogens were inoculated from slants to about 5 ml MRS broths and incubated for 24 h at 30°C. About 1% of the 24 h culture of the pathogen was suspended in 9 mL of normal saline and adjusted to 0.5 McFarland standards. A lawn of the indicator strains were then made by spreading the cell suspension over the surface of Muller Hinton agar plates with a sterile cotton swab. The plates were allowed to dry and a sterile glass rod of diameter 3 mm was used to bore uniform wells in the agar. Each well was then filled with 10 µL of the different cell-free supernatant (LAB culture filtrate). After incubation at 37°C for 24 h, the plates were observed for a zone of inhibition around the well. The antimicrobial activity was expressed as the diameter of the inhibition zones around the wells. Results were considered positive if the diameter of the zones of inhibition were greater than or equal to 4 mm. The negative control used was sterile distilled water while the positive control used were 100 IU of diluted Ciprofloxacin and Azithromycin.

RESULTS AND DISCUSSION

Characteristics of samples

The measurement of the pH shows that corn-beer is more acidic in nature with a pH range of 6.18 at collection time to 4.32 after 48 h fermentation; against 6.51 to 4.66 for palm-wine. However, palm wine taste more sour than corn-beer. This may be because corn-beer is richer in moulds and yeasts than palm wine (Ogbonnaya and Bernice, 2012). These microorganisms may produce organic acid which might have an influence on the souring taste of the corn-beer.

The lactic acid bacteria population in corn-beer ranges from 1.2×10^7 to 6.7×10^7 CFU/mL as against 2.5×10^7 to 7.5×10^7 CFU/ml for palm-wine. Thus, palm-wine is richer in LAB population than corn-beer. The sludge of corn-beer and the sap of the palm tree have been shown to be a rich medium capable of supporting the growth of various types of microorganisms. The dominant populations of microorganisms include aerobic meso-philic bacteria, yeasts, moulds and lactic acid bacteria (Chandrasekhar et al., 2012; Parveens and Hafiz, 2003).

Table 1. Distribution of lactic acid bacteria in palm-wine and corn beer samples.

Isolate	Palm wine	Corn beer
	Frequency (%)	Frequency (%)
L. fermentum	0 (0.0)	2 (10.5)
L. plantarum	3 (21.4)	3 (15.8)
L. mesenteroides	3 (21.4)	3 (15.8)
L. brevis	3 (21.4)	3 (15.8)
L. carnosum	3 (21.4)	1 (5.3)
L. acidophilus	0 (0.0)	2 (10.5)
L. bulgaricus	0 (0.0)	1 (5.3)
P. acidilactici	0 (0.0)	4 (21.1)
L. pentosus	2 (14.3)	0 (0.0)
Total	14 (100)	19 (100)

Frequency = number of the strain/number of isolates x100.

Isolation, characterization and identification of LAB

Following culture on MRS agar, a total of thirty-three isolates Gram positive and catalase negative rods, cocci or tetrads were obtained from palm-wine and corn-beer, as presumptive lactic acid bacteria. Of this number, fourteen were from palm-wine and nineteen from corn-beer. The isolates from corn-beer were coded Cb1, Cb2, Cb3, Cb4, Cb5, Cb6, Cb7, Cb8, Cb9, Cb10, Cb11, Cb12, Cb13, Cb14, Cb15, Cb16, Cb17, Cb18 and Cb19; while those from palm wine were coded Pw1, Pw2, Pw3, Pw4, Pw5, Pw6, Pw7, Pw8, Pw9, Pw10, Pw11, Pw12, Pw13 and Pw14. These isolates were further biochemically characterized and identified using the API 50 CHL Kit (BioMerieux, France).

The nineteen catalase negative isolates obtained from corn beer were identified using API 50 CHL BioMerieux Kit. They were identified as *Lactobacillus plantarum* (Cb1*,* Cb2, Cb3, Cb8), *Lactobacillus brevis* (Cb5, Cb12, Cb19)*, Leuconostoc mesenteroides* (Cb4, Cb6, CB15) and *Leuconostoc carnosum* (Cb7); *Pediococcus acidilactici* (Cb9, Cb11, Cb13, Cb17), *Lactobacillus fermentum* (Cb10), *Lactobacillus acidophilus* (Cb14, Cb16), *Lactobacillus bulgaricus* (C18) while the fourteen (14) LAB isolates from corn-beer, were found to consist of mainly 5 different species namely*, L. plantarum* (Pw2, Pw5, Pw11) , *L. brevis* (Pw1, Pw12, Pw14)*, L. mesenteroides* (Pw3, Pw9, Pw13)*, L. carnosum* (Pw4, Pw7, Pw10)*, L. pentosus* (Pw6, Pw8)*.* The distribution of the LAB isolates is given in Table 1.

Nineteen presumptive LAB isolates were obtained from corn-beer samples from which 8 different LAB species (*L. fermentum, L. plantarum, L. mesenteroides, L. brevis, L. Carnosum, P. acidilactici, L. acidophilus* and *L. bulgaricus*) were identified (Table 1). Meanwhile fourteen (14) presumptive LAB isolates were obtained from palm-wine samples from which 5 different LAB species (*L. brevis, L. plantarum, L. mesenteroides, L. carnosum* and *L. pentosus*) were identified (Table 1). This is somehow contradictory to the fact that palm-wine is richer in LAB population. However, it can be concluded that corn-beer is richer in the content of LAB isolates than palm wine while palm wine is richer in LAB population than corn beer. Four LAB species namely: *L. plantarum, L. mesenteroides, L. brevis* and *L. carnosum* were commonly isolated from both corn-beer and palm-wine samples. However, four other LAB species (*P. acidilactici, L. acidophilus and L. bulgaricus*) were isolated only from corn-beer, while just one LAB species (*L. pentosus*) was isolated only in palm-wine samples. Thus, there is some basic similarity in the content of LAB species found in both beverages.

It can be observed from Tables 1 and 2 that lactobacilli and leuconostocs were the sole lactic acid bacteria isolated from palm wine samples, with lactobacilli being the predominant LAB (57.1%). Lactobacilli are also the predominant LAB in corn-beer (57.9%). This result is similar to the finding of Nwachukwu et al. (2010). They have isolated similar species in traditional weaning food called “Ogi” in Nigeria

Determination of antimicrobial activity

Figure 1 shows the plate assays for inhibitory activity of some LAB isolates against indicator food borne pathogens. All the LAB isolates from corn-beer inhibited the indicator food-borne bacterial pathogens to some degree. The inhibitory activity and the MICs are summarized in Figures 2 and 3, respectively. *Pediococcus acidilactici* manifested a very remarkable antimicrobial effect on the tested food-borne pathogens with mean zones of inhibition of 19, 20 and 16 mm; and MICs of 64, 64 and 32 mg/ml (Figure 3), on *E. coli, S. typhi* and *S. aureus,* respectively. These LAB species were obtained from samples of all the towns in Fako Division with no significant difference in their zone of inhibition. These results were similar to those obtained by Lee et al. (2013). These authors reported the ability of LAB such as *L. rhamnosus* and *L. lactis* to inhibit *Clostridium difficile*.

On the other hand, all the LAB isolates from palm-wine equally inhibited all the food-borne bacterial pathogens tested, by very significant degrees (Figure 2). *L. pentosus* manifested the most remarkable antimicrobial effect from these palm wine samples with mean zones of inhibition of 15, 17 and 15 mm (Figure 2); and MICs of 32, 64 and 32 mg/ml (Figure 3), on *E. coli*, *S. typhi* and *S. aureus,* respectively. These LAB species were obtained only from palm-wine samples from Tiko and Buea and there was no significance difference in their zones of inhibition on the indicator pathogens.

Meanwhile, *L. carnosum* from corn-beer samples manifested the least antimicrobial inhibitory potential on the tests organism, while *L. brevis* from palm-wine manifested the least. These two LAB species are found in

Table 2. Inhibitory substances of the LAB isolates from corn-beer by zones of inhibition.

Bacteria	Inhibition zone on the indicator food borne pathogen (mm)								
	E. coli			*S. typhi*			*S. aureus*		
	B	H	A	B	H	A	B	H	A
L. fermentum	7	8	-	5	6	4	8	6	5
L. plantarum	9	6	-	6	4	-	8	6	-
Lc. mesenteroides	-	8	5	4	14	-	10	4	-
L. brevis	8	6	-	7	5	4	7	4	-
L. carnosum	11	-	-	7	4	-	-	4	6
P. acidilactici	12	-	6	4	-	15	4	-	4
L. acidophilus	4	4	5	10	5	-	5	-	4
L. bulgaricus	8	-	4	6	7	-	8	7	-
Total	59	32	20	49	45	23	50	31	19
Percentage	50.86	27.59	17.24	40.83	37.50	19.17	49.50	30.70	18.81
Missing (%)		4.31			2.5			0.99	

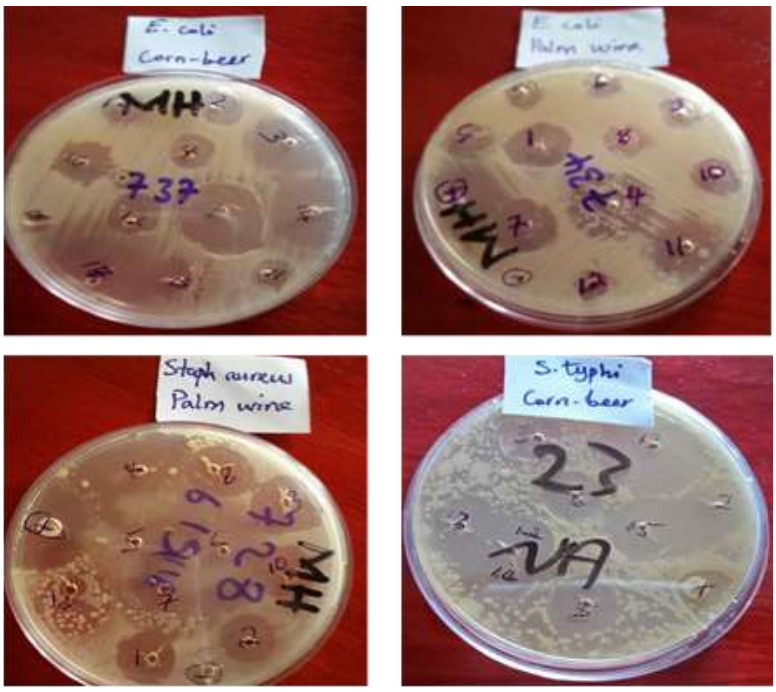

Figure 1. Plate assays for inhibitory activity of lactic acid bacteria on potential foodborne pathogens determined by well diffusion methods.

born corn-beer and palm-wine.

Generally, LABs from corn-beer have more antimicrobial inhibitory potential on food-borne bacterial pathogens than LABs from palm-wine. However, the least inhibitory potential on food borne pathogens was observed from corn-beer and was impacted by *L. acidophilus* (07 mm) and *L. carnosum* (9 mm) on *S. aureus*.

The most susceptible food-borne pathogen to LAB antimicrobial from corn-beer and palm-wine was *S. typhi*. This is interesting because *Salmonella* sp. has been reported as one of the leading cause of illnesses due to food-borne pathogens, and the pathogen has become very resistant to many antibiotics in Cameroon (Akoachere et al., 2009). Therefore, antimicrobials produced by lactic acid bacteria from corn-beer and palm-wine can be exploited for the treatment and prevention of *S. typhi* infections. On the other hand, the least susceptible food-borne pathogen is *S. aureus*. This is of little worrying because, since the emergence of

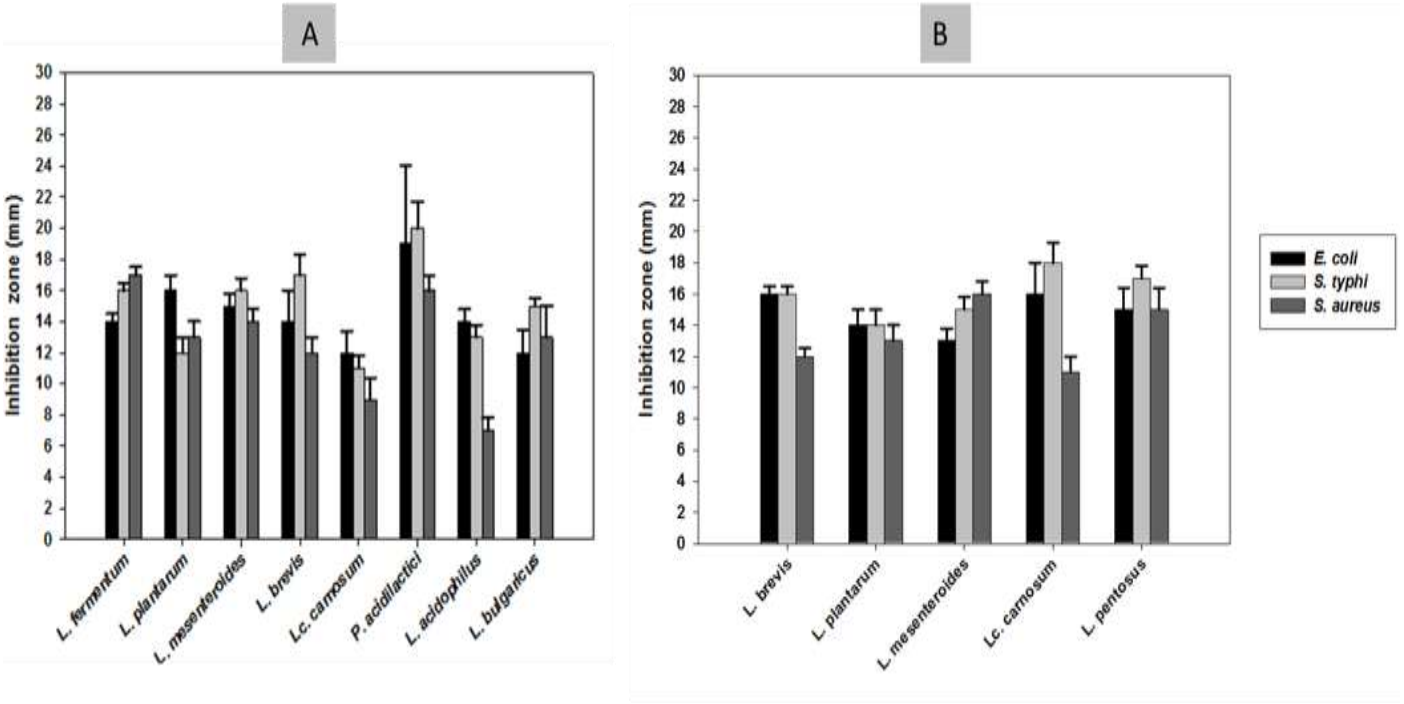

Figure 2. Inhibitory activity of LAB against food borne pathogens (A) corn beer isolates, (B) palm wine isolates. The data shown are averages of triplicate assays with SD within 10% of mean value.

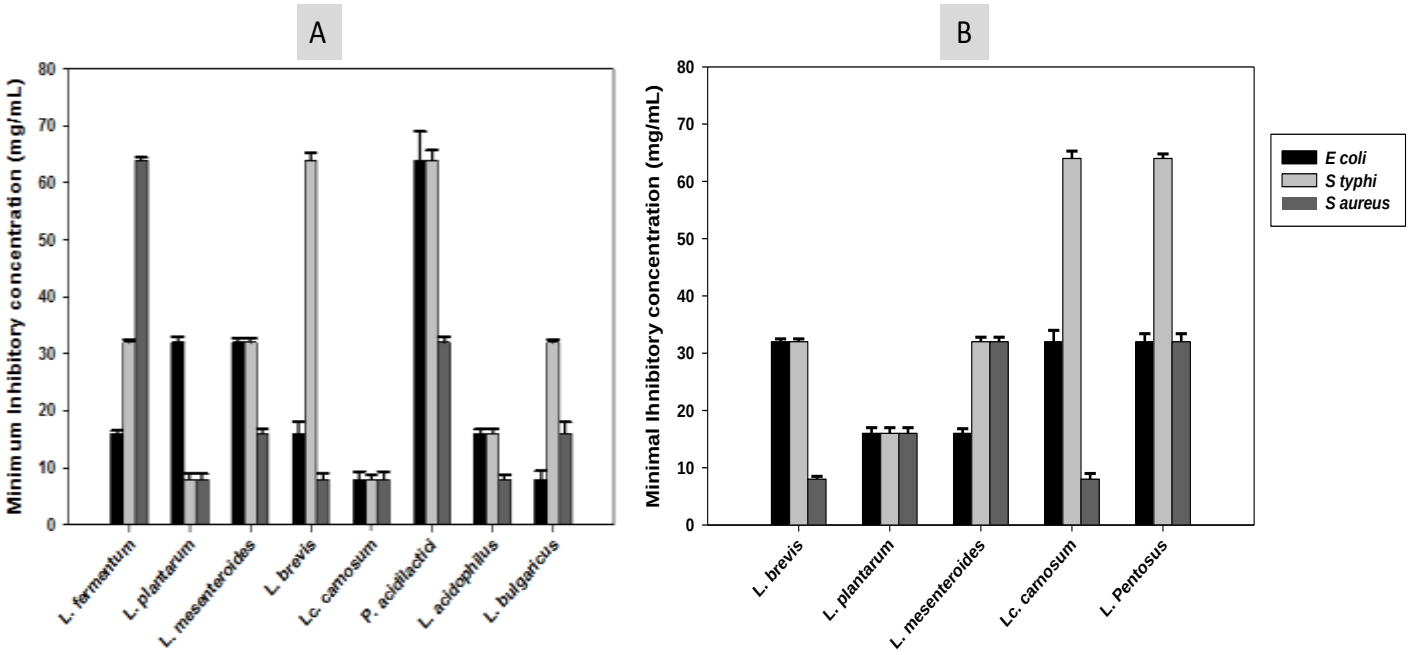

Figure 3. Minimal Inhibitory concentration of supernatant of lactic acid bacteria cultured in MRS broth: (A) Corn beer isolates, (B) palm wine isolates. The data shown are averages of triplicate assays with SD within 10% of mean value.

methicillin resistant *S. aureus* (MRSA), *Staphylococcus* has become resistant to many antibiotics. The inhibitory properties of LAB antimicrobials would have been a reliable candidate to look up in an attempt to cub

Table 3. Inhibitory substances of the LAB isolates from palm-wine.

Bacteria	Inhibition zone on the indicator food borne pathogen (mm)								
	E. coli			*S. typhi*			*S. aureus*		
	B	H	A	B	H	A	B	H	A
L. brevis	9	7	-	4	6	4	6	7	-
L. plantarum	8	4	-	8	6	-	7	5	-
Lc. mesenteroides	-	9	7	6	8	-	10	4	-
L. carnosum	14	-	-	10	7	-	-	-	9
L. pentosus	4	7	4	6	8	6	4	6	8
Total	35	27	11	34	35	10	27	22	17
Percentage (%)	47.30	36.49	14.90	42.5	43.75	12.5	40.30	32.84	25.37
Missing (%)	1.31	1.25	1.49						

Staphylococcal infections but this does not look very promising. However, *S. aureus* was greatly inhibited by antimicrobial from *L. fermentum* (17 mm).

Determination of the inhibitory substance of the LAB isolates

All the LAB isolates inhibited the indicator food borne pathogens tested to some degree by one or two or all of the tested antimicrobial substances namely: Bacteriocins (B), hydrogen peroxide (H) and organic acids (A) (Tables 2 and 3). However, the LAB isolates from corn-beer inhibited the majority of the pathogens by mostly their bacteriocins (Table 2), while the LAB isolates from palm-wine inhibited the indicator pathogens by mostly their bacteriocins and hydrogen peroxide (Table 3). Acid exerted the least inhibitory action on the test pathogens. Since LAB samples showed a high acidity, the real effect of the acid on the indicator pathogens might have been lost in the initial medium in which the LABs were contained. The missing percentages suggest that other factors may contribute to inhibition of the food-borne pathogens, rather than just bacteriocin, hydrogen peroxide and acid. Example of such factors may be bacteriophages. The missing value may also be due to the loss in the synergetic effect of the inhibitory substances by the study methodology.

Following the profound antimicrobial effect exerted by LAB isolates on indicator pathogens, and the determined antimicrobial substances to that effect, the purification of antimicrobial substance produced by LAB from corn-beer and palm-wine can be of great medicinal value to the fight against food-borne pathogens. It is worth noting that bacteriocins from LAB are Generally Regarded as Safe (GRAS) (O'Shea et al., 2013).

Conclusion

The findings obtained in this study showed that traditionally processed corn-beer and palm-wine, are particularly rich in their content of lactic acid bacteria whose antimicrobial properties can be exploited in the fight against human diseases especially food-borne pathogenic bacteria. Bacteriocins were the most common inhibitory substance utilized by the LAB isolates from corn-beer and palm-wine to inhibit food borne pathogens. *S. typhi* appeared to be the most susceptible food-borne bacterial pathogen to the LAB. Therefore, corn-beer and palm-wine obtained from Fako division of Cameroon can display great potentials for the development of antimicrobial against bacterial food borne pathogens.

Conflicts of interests

The authors have not declared any conflict of interests.

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