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Evaluation of Organic Acids, Anti-Salmonella Activities of Lactic Acid Bacteria Isolated from Nigerian Grown Salad Vegetables

Bamidele Tajudeen Akanji[1,2*] and Adeniyi Bolanle Alake[2]

[1]Molecular Biology and Biotechnology Division, Nigerian Institute of Medical Research, Yaba Lagos, Nigeria.
[2]Department of Pharmaceutical Microbiology, Faculty of Pharmacy, University of Ibadan, Ibadan, Nigeria.

Authors' contributions

This work was carried out in collaboration between both authors. Author ABA conceived the work. Author BTA designed, made literature search and carried out the work. Authors ABA and BTA prepared the manuscript for publication. Both authors read and approved the final manuscript.

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ABSTRACT

Aim: To quantitate lactic and acetic acids produced by lactic acid bacteria (LAB) isolated from Nigerian grown salad vegetables and investigate their *in vitro* activities against *Salmonella enterica* ser Typhi.
Study Design: Two group non randomized experimental design.
Place and Duration of Study: Nigerian Institute of Medical Research, Molecular Biology & Biotechnology Division between January 2014 and December 2014.
Methodology: LAB isolated from cabbage, carrot, cucumber and lettuce were identified phenotypically and by partial sequencing of their 16S rRNA genes. Twenty three were investigated for the abilities to produce lactic and acetic acids in growth medium by high performance liquid chromatography following standard protocols. The anti-salmonella activities were performed using acidic and neutralized cell free supernatants (CFS) in agar well diffusion assay according to Clinical

**Corresponding author: Email: bamideletj@gmail.com; deletaju@yahoo.co.uk;*

Laboratory Standard Institute guidelines. The differences in concentrations between the organic acids were determined by pair t- test at 95% confidence interval.

Results: The isolated LAB were *Lactobacillus spp*, *Weissella spp*, *Pediococcus spp*, Uncultured solibacillus RBL-135 and *Enterococcus durans* RO2-22. They produced the organic acids at varying degrees with significant difference in concentrations ($P< .05$); *E. durans* RO2-22 from lettuce produced 52483 µg/ml and 30439 µg/ml followed by *W. confusa* FS027 from cabbage producing 20480 µg/ml and 17184µg/ml of lactic and acetic acids respectively. The lowest (134 µg/ml and 517 µg/ml), was produced by *L. johnsonii* MH8 from lettuce. Three, *L. johnsonii* MH8, *W. confusa* SJL 602 from lettuce and *W. confusa* FS027 from cabbage produced more acetic than lactic acid.

The acidic CFS of *W. cibaria*, *W. confusa* showed between 15-16 mm zones of inhibition while *P. pentosaceus*, *P. acidilactici*, *L. plantarum* and *L. fermentum* showed the least (12- 13 mm).

Conclusion: Most of the selected LAB were able to produce significantly more lactic than acetic acid and their inhibitory activities against the test pathogen was mainly due to low pH.

Keywords: Organic acids; lactic acid bacteria; high performance liquid chromatography; Salmonella enterica ser Typhi; polymerase chain reaction; cell free supernatant.

1. INTRODUCTION

Lactic acid bacteria (LAB) have a long and safe history of use as anti-infectious agents and as food adjuncts. Their antagonistic activities are due to the effects of metabolites produced such as organic acids (lactic, acetic), hydrogen peroxide, diacetyl and bacteriocins et cetera.

Lactic and acetic acids produced by LAB are often responsible for low pH in the medium, taste/ odour enhancements. Lactic acid is considered to be one of the most useful chemicals. It is applied in the industries as food preservative, accidulant and flavouring in textile and pharmaceutical industries, source of lactate ester, propulene glycol, 2, 3-pentanedione, propanoic acid, acrylic acid, acetaldehyde and dilactide [1,2] in chemical industries. It is also a well known monomer in the production of poly lactic acid (PLA) which is sustainable bioplastic material [3,4]. Acetic acid on the other hand is employed in industrial fermentation to produce vinegar.

The inhibitory activities of LAB against *Salmonella spp* have been variously reported including adherence to human GIT where LAB tend to outcompete the pathogens in their adherence to GIT. For instance, adherent live or heat killed *L. acidophilus* LB and live *L. johnsonii* La1 bacteria in the presence of spent culture supernatant exhibited dose- dependent inhibition of adhesion to the brush of Caco-2 cells by *S. enterica* ser Typhimurium in a concentration-dependent manner [5-7].

In same vein, *L. casei* rhamnosus GG and *L. casei* Shirota were able to compete with *Salmonella* spp probably by means of steric hinderance even as human LAB strains, *L. acidophilus* and *L. fermentum* from chicken intestine were able to produce exclusion and competition in cultured intestinal cells if they were infected by *S. typhimurium* [8].

The most common infectious model used to investigate the antibacterial activity of LAB is that of gnotobiotic or conventional mice infected by *S. enterica* ser Typhimurium, *L. johnsonii* La1 and GG strains [9,10], which colonize the gut of gnotobiotic C3H/ He/ Oujco mice, developed anti- salmonella activity when the mice were orally infected by *S. enterica* ser Typhimurium C5, increasing the survival of mice even as *L. rhamnosus* HN001 has the ability to confer immune enhancement and protection to BALB/c mice orally challenged with this pathogen [11]. In the axenic C3/He/Oujco mouse, human *Bifidobacterium* spp, CA1 and F9 bacteria colonized the intestinal tract and protected the mice against a lethal infection of this *S. enterica* C5, which suggest that they could contribute to the "barrier effect" produced by the indigenous mocrobiota [12].

B. lactis HN019 in BALB/c mice also conferred protection against this pathogen, which included a 10- fold increase in the survival rate, a significantly higher post- challenge food intake and weight gain, and reduced translocation of the pathogen to the spleen and liver [13]. *B. breve* Yakult exhibited anti- infectious activity against *S. enterica* when opportunistic, antibiotic-induced murine infection model was used [14]. In contrast however, *B. bifidum* ATCC 15696 and *B. catenulatum* ATCC 27539T strains have no

effect even with high population levels similar to those of the *B. breve* strain.

LAB isolated from fresh vegetables and fruits such as lettuce have also been reported to show anti- salmonella activities; Trias et al. [15] documented *L. plantarum*, *Leuconostoc spp* and *Weissella spp* as being antagonistic to *S. typhimurium* when the vegetable wounds were treated with these LAB, while [16] used LAB culture condensate mixture (LCCM) to inhibit *S. enteritidis* both in vitro and in mice.

In Nigeria, there is paucity of information on the quantum of organic acids produced by LAB isolated from salad vegetables. This study evaluated the quantities of lactic and acetic acids produced by LAB from these vegetables and also investigated their *in vitro* anti- salmonella activities.

2. MATERIALS AND METHODS

2.1 Study Design

Two group non randomized experimental design was employed. This involved the test group which is the inoculated cultures and control.

2.1.1 Processing of salad vegetables

Cabbage, carrot, cucumber and lettuce were processed according to standard methods; they were blended aseptically, transferred to sterile de Man Rogosa Sharpe (MRS) broth and incubated micraerophilically at 37°C for 24 h.

2.1.1.1 Isolation of LAB

Inoculum was streaked into sterile MRS agar plates and incubated further as above. The distinct colonies were tested for catalase, oxidase (Oxoid, UK), spore forming and Gram stain reactions following standard methods.

2.1.1.2 Identification of LAB

All Gram positive, catalase, oxidase negative isolates without spores were subjected to Polymerase Chain Reaction (PCR) of their 16S rRNA gene as follows.

2.1.1.3 DNA extraction

This was done using Zymo Research Fungal/ Bacterial DNA mini prep (USA) kit following the manufacturers' protocols. The extracts were quantitated and ascertained for purity using Nano drop spectrophotometric instrument (ND- 1000, Thermo- Scientific, USA).

2.1.1.4 Amplification of 16S rRNA gene

The set of primers (0.2 µl each); BSF- 8(51-AGAGTTTGATCCTGGCTCAG-31) and BSR-534 (51- ATTACCGCGGCTGCTGGC- 31) were employed [17] and 4 µl of 5X HOT Firepol mastermix (Solis biodyne, Estonia) added to each PCR reaction tube in which 2 µl of template DNA has been added. The reaction mixture was made up to 20 µl with DNAse free distilled water. The PCR was carried out following the cycle parameters for the primers.

2.1.1.5 Agarose gel electrophoresis

The PCR product (5 µl) was loaded alongside a 100bp marker (Thermo Scientific, USA) on a 1% agarose stained with ethidium bromide (10 µl of 1 mg/ml) and electrophesis run for 1 hr at 100 V (Bio-Rad, USA). The gel was viewed under ultraviolet (UV) ray in a gel photodocumentation system (Clinix, China).

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

2.1.1.6 Centrifugation of MRS broth culture

This was done at 4°C using Eppend off centrifuge 5702R at 10,000 g for 10 min. The supernatant was separated and membrane filtered (Millipore, 0.22 µm) and the filterates (cell free supernatant, CFS) used for anti-Salmonella assays and quantitation of organic acids.

2.1.1.7 Quantitation of organic acids

Lactic and acetic acids were assayed for, using protein precipitation techniques (liquid- liquid extraction) and the analysis done by high performance liquid chromatography 1200 series (HPLC Agilent, USA).

The preparation of standard solutions for calibration curve, chromatographic conditions were done following standard protocols. The peak areas (mAU) were plotted against standard concentration (µg/ml) to produce standard regression (scatter plot) graph (Figs. 1 & 2).

The sample concentrations were calculated from the regression line equation, y= mx+ c where y= peak areas (mAU), m= slope, x= concentration (µg/ml) and c= intercept on the y- axis.

2.1.1.8 Anti- salmonella assay

The test pathogen, *Salmonella enterica* ser Typhi previously isolated from food handler was supplied by the culture bank of Nigerian Institute of Medical research (NIMR), Nigeria. This assay was performed as follows; 100 µl of the CFS was introduced into well bored (using sterile cork borer, 6 mm diameter) on Mueller Hinton agar which has been seeded with sterile normal saline suspended, 0.5 Mc Farland standard equivalent to 10^8 colony forming unit per millilitre (CFU/ml) of the test pathogen. This was incubated in air atmosphere at 37°C for 24 hrs after which the zones of inhibition were measured in millimeter. The negative control comprising of sterile MRS broth incubated in same environment as cultures was also used in this assay.

2.2 Statistical Analysis

Using IBM SPSS vs 20, a confirmation of normality of the data was done before conducting a pair t- test to investigate differences between the concentrations of lactic and acetic acid produced by each LAB at 95% Confidence interval.

3. RESULTS

3.1 Identification of LAB

Ninety- two of the PCR products of 115 LAB isolates loaded for agarose gel electrophoresis amplified the gene corresponding to 526 bp in size. The amplified gene gave between 93-99% similarities to 16 different LAB spp in GeneBank as follows; *Lactobacillus spp* (8), *Weissella spp* (3), *Pediococcus* spp (3), uncultured solibacillus RBL-135 (1) and *Enterococcus durans* RO2-22 (1) – (Table 1).

3.2 Quantitation of Organic Acids

Twenty- three LAB was randomly selected for this assay based on their abilities to inhibit the study pathogen.

All these produced lactic and acetic acids at varying degrees (between 4 to 5 digits) except *L. johnsonii* MH8 which produced 134 µl/ ml lactic acid and 517 µl/ ml acetic acid. *Enterococcus durans* R02- 22 isolated from lettuce, produced the highest amounts of both acids (52483 µg/ml and 30439 µg/ml of lactic and acetic respectively) followed by *W. confusa* FS027 from cabbage producing 20480 µg/ml and 17184 µg/ml of lactic and acetic acids respectively (Table 2). It was also indicated that most of the respective LAB tended to produce more of lactic rather than acetic acid (Figs. 3 & 4). The concentrations of the organic acids were normally distributed when tested before applying t- test. The difference in concentrations of lactic and acetic acids produced by the LAB was statistically significant ($p< 0.05$).

3.2.1 Anti- salmonella assay

The CFS of LAB showed varied degrees of inhibition against *Salmonella enterica* ser Typhi. The widest (16 mm) was seen in *W. cibaria* isolated from cabbage and cucumber followed closely (15 mm) by *W. confusa* from cabbage, cucumber and lettuce respectively (Table 3). *Pediococcus pentosaceus* from cabbage, *P. acidilactici*, *W. paramesenteroides* and *L. fermentum* from cucumber showed inhibition of 14 mm each. The narrowest inhibition (12 mm) was seen in *P. pentosaceus* from cucmber and lettuce, *P. acidilactici*, *L. fermentum* both from lettuce, *L. plantarum* from cabbage and cucumber respectively (Table 3).

4. DISCUSSION

The occurrence of the LAB in cabbage, carrot, cucumber and cabbage in the study sites and their antibacterial activities have been previously reported [18,19]. In the studies their activities against pathogens such as *P. aeruginosa* ATCC 27853, *E. faecalis* ATCC 29212, *E. coli* ATCC 12900, *Proteus penneri* ATCC 13315 and outbreak strain of *V. cholerae* O1 were reported. In this report, the killing abilities of the LAB against *S. enterica* isolated from food handlers were evaluated.

The anti- salmonella activities of LAB against *S. enterica* was demonstrated by [20] and recently, [21] reported the inhibitory activities of selected *lactobacilli* species against Gram positive, Gram negative bacterial and yeast pathogens.

The findings by [22] showed that some bacteriocin producing LAB strains were not able to inhibit both *Salmonella spp* and *V. cholerae* when the effect of acid was excluded.

In this study, although neutralized CFS from 2 LAB (*P. pentosaceus, L. fermentum* from lettuce and cucumber respectively) showed marginal inhibitions of between 7-10 mm diameter while the third, *L. fermentum* from cucumber maintained same inhibition zone of 14 mm as in acidic CFS. The Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS- PAGE) assay done on these CFS did not indicate production of proteinaceous substances (unpublished data). The anti- salmonella activities of the LAB were mainly due to the effect of organic acid. These inhibitions seen after excluding the effect of organic acids might have been due to hydrogen peroxide.

The neutralized CFS (from the 2 LAB) producing a reduction in zones of inhibition is in tandem to the study of [20], that demonstrated a synergistic effect of organic acids and hydrogen peroxide produced by LAB against different pathogens out of which was *S. enterica* in a co- culture system.

The innate ability of the isolated LAB to produce bacteriocins however cannot be ruled out completely as certain cultural/ environmental parameters if put in place, can ginger the production. The screening for bacteriocins production can be carried out using specific primers for this purpose in PCR assay.

Table 1. Summary of LAB isolated from salad vegetables

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

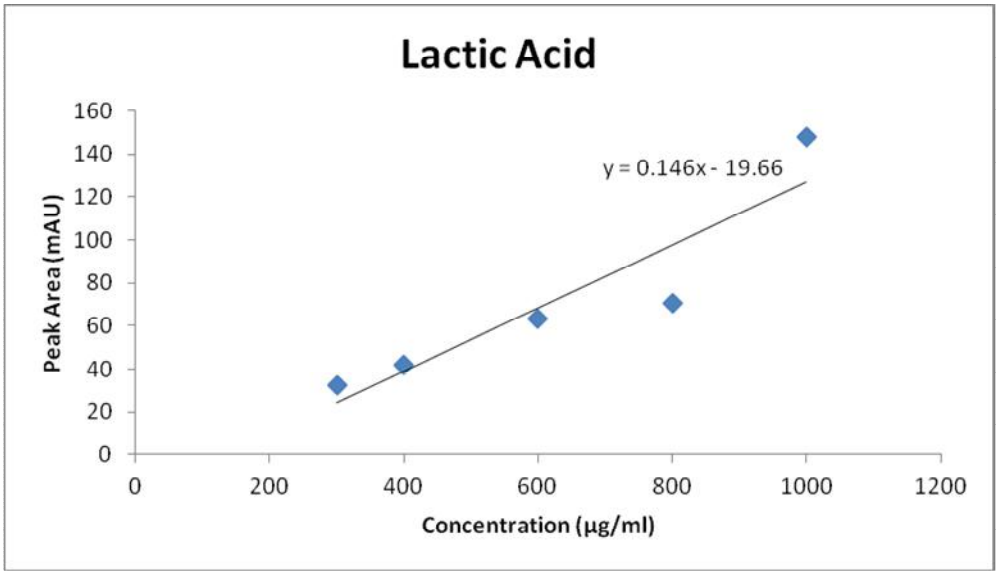

Fig. 1. Standard regression plot for lactic acid

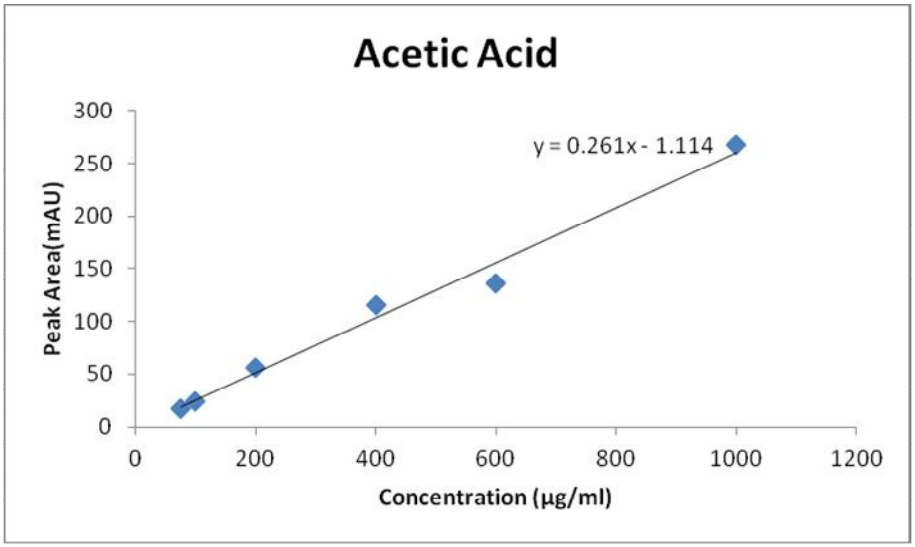

Fig. 2. Standard regression plot for acetic acid

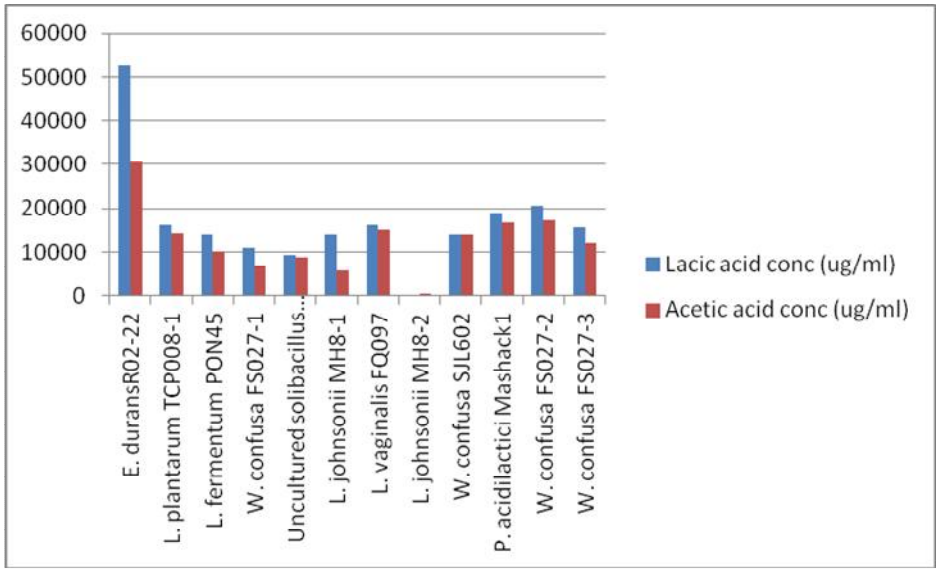

Fig. 3. Concentrations of lactic and acetic acid produced by LAB strains

Lactic acid bacteria produce a variety of metabolites like organic acids (Lactic, acetic), hydrogen peroxide, diacetyl, bacteriocins et cetera that can inhibit closely related, unrelated bacteria and even fungi. Bacteriocins have particularly been found to be responsible for different inhibitions. In this study, the antagonism of the test pathogens was mainly due to the effect of low pH of the CFS.

The results of organic acids quantitation by HPLC in this study indicated that the LAB has the abilities to produce both acids (lactic and acetic) at varying degrees.

The production of these acids in quantities and trend was in partial agreement with the work of [23] in Nigeria where *L. plantarum* and *L. brevis* isolated from fermentating millet gruel reportedly produced 1.80 g/l and 0.63 g/l lactic acid respectively.

In the present study, highly sensitive chromatographic method, HPLC was used to quantify the acids while the titration methods were employed by [23]. *L. plantarum* TCP008 isolated from lettuce and *L. brevis* BB7 from cucumber in this study produced 0.02 g/l each.

Table 2. Lactic and acetic acids concentration (µg/ml) produced by LAB

LAB Strains (vegetable source)	Lactic acid concentration	Acetic acid concentration
E. durans R02-22 (Lettuce)	52483.30461	30439.80155
L. plantarum TCP008 (Lettuce)	16224.30031	14128.0539
L. fermentum PON45 (Cabbage)	13894.41864	10224.56985
W. confusa FS027 (Lettuce)	11050.2813	6823.051952
Uncultured solibacillus Clone RBL-135 (Cucumber)	9343.260703	8605.952289
L. johnsonii MH8 (Lettuce)	13918.42094	5618.919172
L. vaginalis FQ097 (Lettuce)	16267.27968	15001.2981
L. johnsonii MH8 (Lettuce)	134.0939358	517.9942765
W. confusa SJL602 (Lettuce)	13844.71543	14073.23268
P. acidilactici Mashack1 (Cucumber)	19041.43011	16797.68364
W. confusa FS027 (Cabbage)	20480.3407	17184.87344
W. confusa FS027 (Cabbage)	15688.7829	11963.68051
W. confusa FS027 (Carrot)	13328.21846	9930.154776
W. confusa FS027 (Cabbage)	11269.86396	13559.31914
P.pentosaceus FMAC63 (Cucumber)	12483.15476	7129.660613
W. cibaria PON10339 (Cucumber)	11950.34799	5900.068228
L. plantarum TCP008 (Cabbage)	8290.369134	3355.539733
W. paramesenteroides 69DCEPO1MX (Cucumber)	15441.61093	8580.693151
L. plantarum TCP008 (Lettuce)	13251.50934	7353.631321
L. brevis BB7 (Cucumber)	17897.96062	17721.32018
Lactobacillus sp THK- V8 (Cabbage)	16388.33311	7162.532922
P. pentosaceus Wg8 (Carrot)	19406.43298	4945.677731
Lactobacillus sp THK- V8 (Cabbage)	14172.59219	6858.842801

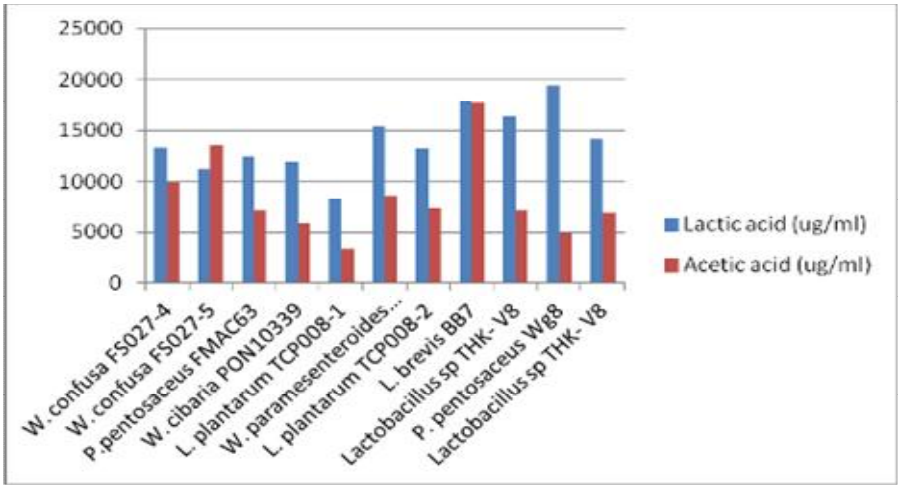

Fig. 4. Concentrations of lactic and acetic acid produced by LAB strains

The inhibitory activities of LAB have been suggested to be directly proportional to the quantum of organic acids produced especially acetic acid. For instance, the CFS of *L. plantarum* from cabbage in this study showed narrowest inhibition (12 mm) against the pathogen. This LAB produced merely 0.008 g/l and 0.003 g/l respectively of lactic and acetic acids. *Weissella confusa* FS027 from cabbage on the other hand, produced 0.02 g/l each of both acids and showed the wider inhibitory zone (15 mm). This trend however, did not reflect in organic acids produced by other LAB.

However, the acidic inhibitory activities of LAB have generally been adduced to acetic rather than lactic acid [24,25]. Largely, the quantity and types of organic acid produced depend on

Table 3. Zones of inhibition of LAB CFS against *S. enterica* ser Typhi

LAB	Frequency	Vegetable	Inhibition zone range (mm)
P. pentosaceus	3	Cucumber	12-13
,,	2	Cabbage	13-14
,,	1	Lettuce	12
P. acidilactici	3	Cucumber	14
,,	2	Lettuce	12
P. dextrinicus	1	Cabbage	13
W. confusa	2	Cucumber	15
,,	5	Cabbage	14-15
,,	4	Lettuce	13-15
W. cibaria	1	Cucumber	16
,,	2	Cabbage	15-16
W. paramesenteroides	1	Cucumber	14
L. plantarum	1	Cucumber	12
,,	1	Cabbage	12
L. fermentum	5	Cucumber	12-14
,,	1	Lettuce	12

cultural/ environmental conditions and lactic acid tends to be in larger quantity than acetic acid [26,27]. This was also seen in this study as same LAB strains from different vegetable market location tended to produce different quantities of organic acid (Locations not shown) and that the LAB generally produced significantly more lactic than acetic acid.

The ability of *E. durans* RO2-22 isolated from lettuce in this study, to produce the highest quantities of both acids is remarkable. The LAB produced approximately 0.1 g/l and 0.03 g/l of lactic and acetic acid respectively.

5. CONCLUSIONS

The LAB from the study salad vegetables can produce both lactic and acetic acids and in particular, *E. durans* RO2-22 produced the highest concentrations of both acids. These can be cheap and biological sources of these acids. The LAB also possessed the killing abilities against *S. enterica* ser Typhi due mainly to their acidic CFS.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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