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# Quantitative estimation of antimicrobials produced by Lactic Acid Bacteria isolated from Nigerian beef

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**Abstract:** The evaluation of five strains of *Pediococcus*, isolated from Nigerian beef in a previous work, for the production of various antimicrobials is being reported in the present study. The objective was to assess the potential of the strains for use as biopreservatives in food preservation techniques, especially meat processing. The strains tested were *Pediococcus pentosaseus* LIV01 (GenBank accession number EU667381), *Ped. acidilactici* FLE01 (Accession number EU667382), *Ped. acidilactici* FLE02 (Accession number EU667385), *Ped. pentosaceus* INT02 (Accession number EU667384), and *Ped. pentosaceus* INT01 (Accession number EU667383). The methods involving high performance liquid chromatography, gas chromatography–mass spectrometry and peroxidise enzyme were adopted to assay for production of the antimicrobials - organic acids, diacetyl and hydrogen peroxides respectively, by the *Pediococcus* strains. All figures obtained for the concentrations of lactic and acetic acids were normalised as g/$10^7$ CFU while those of hydrogen peroxides and diacetyl as µg/$10^7$ CFU. Generally, lactic acid production increased with incubation time for all the isolates; the highest production of 28.02 g/$10^7$ CFU was recorded for *Ped. pentosaceus* INT01, followed by 23.37 g/$10^7$ CFU for *Ped. pentosaceus* INT02 within 42 h. The concentrations of acetic acid produced by the isolates were lower compared to those of lactic acid. Diacetyl was produced in minimal amounts, and the highest concentration of 57.89 µg/$10^7$ CFU was recorded for *Ped. pentosaceus* INT02. It was concluded that the *Pediococcus* strains could serve as promising candidates for use as protective cultures in meat preservation processes, based on their levels of production of the various antimicrobials. The higher production of diacetyl by *Ped. pentosaceus* INT02, in comparison to other strains, may be of particular interest when selecting candidates as biopreservatives for food bioprocessing.

**Keywords: Antimicrobials, biopreservatives, organic acids, diacetyl, protective cultures, meat preservation**

## Introduction

The genus *Pediococcus* is among the groups of lactic acid bacteria (LAB), which have long been known to produce antimicrobial agents that play vital roles in food fermentation processes, and hence are very useful in preservation of many food items (Olaoye, 2009). The primary antimicrobial effect exerted by LAB is the production of lactic acid and reduction of pH and the levels of production of organic acids by LAB depend on species or strain, culture composition and growth conditions (Lindgren and Dobrogosz, 1990). In addition, they produce various low-molecular-mass compounds such as hydrogen peroxide ($H_2O_2$), carbon dioxide ($CO_2$), diacetyl (2,3-butanedione), uncharacterized compounds, and high-molecular-mass compounds like bacteriocins. All of these can antagonize the growth of some spoilage and pathogenic bacteria in foods and have been explored in the control of most unwanted organisms, but their production is variable amongst strains of LAB. The aim of the work presented in this report was to evaluate the production of these antimicrobials amongst the some *Pediococcus* strains, whose isolation from Nigerian beef has been reported previously (Olaoye *et al*., 2008). This could be very important in assessing their suitability as protective cultures in the biopreservation of meat and other food products.

## Materials and Methods

### *Source of Lactic Acid Bacteria and culture conditions*

The lactic acid bacterial cultures used consisted of five isolates that have been isolated and identified from Nigerian beef in a previous study (Olaoye *et al*., 2008). Their growth conditions are shown in Table 1.

### *Quantitative estimation of organic acids using high performance liquid chromatography (HPLC)*

A method based on HPLC as described by Olaoye *et al*. (2008) was used to assay for the organic

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**Table 1.** Lactic acid bacterial strains and culture condition

| Name | GenBank Accession no | Media | Temp (°C) | Source |
|---|---|---|---|---|
| *Pediococcus pentosaceus* LIV01 | EU667381 | MRS | 30 | Olaoye *et al*., 2008 |
| *Ped. acidilactici* FLE01 | EU667382 | MRS | 30 | Olaoye *et al*., 2008 |
| *Ped. acidilactici* FLE02 | EU667385 | MRS | 30 | Olaoye *et al*., 2008 |
| *Ped. pentosaceus* INT01 | EU667383 | MRS | 30 | Olaoye *et al*., 2008 |
| *Ped. pentosaceus* INT02 | EU667384 | MRS | 30 | Olaoye *et al*., 2008 |

acids produced by the *Pediococcus* isolates *in vitro*, with modifications. The full details of the modified method are described as follows. An inoculum of a *Pediococcus* isolate (100 µl each, representing ~ $10^6$ cfu/ml) of 24 h MRS broth cultures (grown at 30°C), adjusted to same optical density, was transferred into a 30 ml sterile MRS broth in universal bottle, and incubated at 30°C in a shaking incubator (200 rpm) for 48 h. At 6 h intervals, 15 ml of sample was removed and centrifuged at 3,500 x g for 15 min. The cell free supernatant (CFS) was obtained and filter sterilized by passing through a 0.2 µm syringe filter (Sartorius AG 37070 Goettingen, Germany).

The organic acids were extracted from the CFS using the following procedure. The Cartridge (Strata X 33u Polymeric Reversed Phase, 30 mg/ml, Phenomenex, UK) was conditioned by slowly passing 1 ml of absolute methanol (HPLC grade) through, followed by 1 ml of 10% (v/v) methanol in $NaH_2PO_4$:Methanol, 98:2 (Fernandez-Garcia and McGregor, 1994). The mixture of the HPLC mobile phase (10% Methanol in $NaH_2PO_4$:Methanol, 98:2) and sample supernatant (in ratio 3:1, i.e 900 µl mobile phase and 300 µl supernatant) was then slowly passed through the pre-conditioned cartridge. Few drops (200-250 µl) were allowed to run off while the remaining (950-1000 µl) was collected in an Eppendorf tube for injection into the HPLC system. Samples for analyses were prepared in three replicates.

Uninoculated MRS broth, prepared as the samples, was used to set a baseline for measurement of the organic acids. Standard concentrations (g/l) of 0.5, 1.0, 2.5, 5.0, 7.5, 10, 12.5, 15, 17.5 and 20 of the lactic and acetic acids were prepared and analysed by HPLC. Results were used to plot standard curves used in measuring the concentrations of the acids in the samples. All figures for the concentrations of lactic and acetic acids have been normalised as g/$10^7$ CFU. The HPLC system and chromatographic conditions used were same as previously described (Zotou *et al*., 2004; Olaoye *et al*., 2008).

*Quantitative determination of hydrogen peroxide using peroxidase enzyme*

This was determined using a modification of the methods of Villegas and Gilland (1998) and Jaroni and Brashears (2000). A colony from an 18 h MRS culture was used to inoculate MRS broth (30 ml) and incubated for 18 h at 37°C, shaking at 200 rpm. The broth culture was then centrifuged at 3,500 x g (Centrifuge: Falcon 6/300 CFC Free, UK) for 10 min and the cells were washed twice in cold sterile sodium phosphate buffer (0.2 M, pH 6.5). The cells were suspended by mixing properly in 5 ml of sterile deionised (DO) and then brought to the same approximate cell density of approximately 1 x $10^9$ cfu/ml at an absorbance of 540 nm with sterile DO, using the Cecil CE 2021 (2000 series) spectrophotometer. Thereafter, 2 ml of the cell suspension was inoculated into 20 ml of sterile sodium phosphate buffer (0.2 M, pH 6.5), with or without 55.5 mM glucose, in 30 ml capacity universal bottles. They were incubated for 48 h, and samples taken at every 6 h to determine $H_2O_2$ concentration, absorbance and viable counts. Samples were centrifuged at 3,500 x g, and 2.5 ml of supernatant was added into a test tube containing a mixture of 0.5 ml 0.1% aqueous solution of peroxidase (Horseradish Type VI-A; Sigma Chemical Co., UK) and 0.05 ml of a 1% aqueous solution of *o*-dianisidine (Sigma). A blank was prepared using 2.5 ml of 0.2 M sodium phosphate buffer. Tubes were incubated for 10 min at 37°C, after which the reaction was stopped by adding 0.1 ml of 4 N HCl to each test reaction. Absorbance (at 400 nm) was measured and hydrogen peroxide content was determined against a standard curve of $H_2O_2$ in sodium phosphate buffer (1-20 µg/ml). All figures for the hydrogen peroxide concentrations have been normalized as µg/$10^7$ CFU.

*Quantitative determination of diacetyl using Gas Chromatography (GC)*

For the production of diacetyl (DA) by the *Pediococcus* strains a method using head-space analysis and gas chromatography–mass spectrometry (GC-MS) was used. Sample preparation was the same as that used for the organic acids to obtain CFS. Based on non-detection of DA in the CFS by the GC during preliminary trials (probably due to low concentration), the CFS was spiked with a known concentration of DA and recovery attempts were then made. A recovery concentration of DA above 100% was assumed to be due to that present in the CFS originally. Preparation of standard concentrations of DA was made in blank MRS, analysed by GC and the results were used to plot a standard graph (Figure 1), which was used to measure the concentrations of DA in the broth supernatants of the *Pediococcus* isolates. Blank MRS broth was used to set a baseline for measuring DA. To the best of our knowledge, this method of assaying for DA in MRS broth is being reported for the first

time, as previous reports on this are not known. The use of GC has been applied to quantify DA from other media, such as cheese (Litopoulou-Tzanetaki and Vafopoulou-Mastrojiannaki, 1988) and wines (Hayasaka and Bartowsky, 1999); application of the methods described by the authors was, however, not successful when used during preliminary trials of the present study.

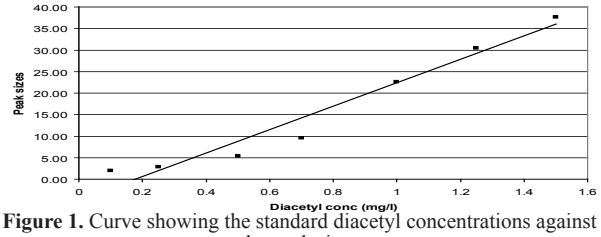


**Figure 1.** Curve showing the standard diacetyl concentrations against the peak sizes

Typical chromatograms generated by the GC during assay for DA are shown in Figure 2. As expected, the peak sizes increased with corresponding increase in concentrations of the DA standards (Figure 1) and the retention time (RT) was approximately 2.85 m (Figure 2; arrowed). Represented in Tables 2 are the peak sizes and DA concentrations obtained after addition (spiking) of 1.0 mg/l to test MRS broth supernatants. The peak size obtained after addition of 1.0 mg/l to blank MRS broth was 22.60. The DA recovery from spiked MRS broth supernatants of the respective *Pediococcus* isolates are shown in Tables 3 and 4. Generally the recovery of peak sizes and DA were greater than 100%, the residual being that generated in the original broth cultures of the isolates. All figures obtained for the diacetyl concentrations have been normalised as µg/$10^7$ CFU.

**Table 2.** Peak sizes and Diacetyl (DA) concentrations after addition of 1.0 mg/l to broth supernatants of *Pediococcus* isolates

| Time (Hrs) | | LIV01 | FLE01 | FLE02 | INT01 | INT02 |
|---|---|---|---|---|---|---|
| 6 | Peak size | 22.74±1.3 | 22.65±2.4 | 22.69±5.01 | 22.71±2.01 | 22.70±0.98 |
| | DA, mg/l | 1.01±0.01 | 1.002±0.03 | 1.005±0.13 | 1.005±0.19 | 1.005±0.02 |
| 12 | Peak size | 22.99±2.3 | 22.93±4.62 | 23.01±3.21 | 23.84±2.71 | 23.72±1.92 |
| | DA, mg/l | 1.02±0.01 | 1.02±0.01 | 1.020±0.11 | 1.050±0.09 | 1.030±0.03 |
| 18 | Peak size | 24.08±0.9 | 23.96±0.91 | 24.37±3.01 | 27.57±2.78 | 26.49±3.91 |
| | DA, mg/l | 1.07±0.04 | 1.05±0.01 | 1.080±0.02 | 1.190±0.11 | 1.15±0.01 |
| 24 | Peak size | 23.61±0.78 | 23.59±2.91 | 23.73±1.99 | 25.78±4.05 | 25.64±4.01 |
| | DA, mg/l | 1.04±0.01 | 1.04±0.01 | 1.050±0.13 | 1.120±0.01 | 1.11±0.01 |
| 30 | Peak size | 23.29±3.01 | 23.31±3.19 | 23.34±2.11 | 24.83±2.61 | 24.77±2.09 |
| | DA, mg/l | 1.03±0.05 | 1.03±0.04 | 1.030±0.01 | 1.090±0.03 | 1.09±0.01 |
| 36 | Peak size | 23.00±4.01 | 22.99±1.97 | 23.01±3.04 | 23.94±3.04 | 23.83±3.75 |
| | DA, mg/l | 1.02±0.01 | 1.02±0.01 | 1.020±0.19 | 1.050±0.06 | 1.05±0.09 |
| 42 | Peak size | 22.87±3.41 | 22.85±2.3 | 22.86±1.97 | 23.58±2.19 | 23.48±2.09 |
| | DA, mg/l | 1.01±0.03 | 1.01±0.06 | 1.010±0.02 | 1.040±0.01 | 1.03±0.14 |
| 48 | Peak size | 22.68±1.01 | 22.69±1.90 | 22.72±3.12 | 23.01±0.99 | 22.95±2.73 |
| | DA, mg/l | 1.005±0.06 | 1.005±0.04 | 1.010±0.01 | 1.020±0.06 | 1.02±0.19 |

LIV01, FLE01, FLE02, INT01, INT02 and are as defined in Table 1. Original Peak size of 1.0mg/l standard DA was 22.60±1.98

**Table 3.** Recovery of diacetyl (µg/l) from broth supernatants of *Pediococcus* isolates

| Time/Isolate | LIV01 | FLE01 | FLE02 | INT01 | INT02 |
|---|---|---|---|---|---|
| 6h | 10±0.32 | 2±0.02 | 5±0.61 | 5±0.91 | 5±0.27 |
| 12h | 20±0.13 | 20±0.63 | 20±0.01 | 50±0.65 | 30±0.71 |
| 18h | 70±1.90 | 50±0.22 | 80±0.99 | 190±2.32 | 150±2.67 |
| 24h | 40±0.21 | 40±0.59 | 50±0.87 | 120±2.76 | 110±1.28 |
| 30h | 30±0.98 | 30±0.79 | 30±0.02 | 90±1.78 | 90±1.27 |
| 36h | 20±0.59 | 20±0.92 | 20±0.39 | 50±0.39 | 50±0.87 |
| 42h | 10±0.83 | 10±0.39 | 10±0.20 | 40±0.47 | 30±0.82 |
| 48h | 5±0.07 | 5±0.27 | 10±0.91 | 20±0.82 | 20±0.88 |

LIV01, FLE01, FLE02, INT01, and INT02 are as defined in Table 1.

**Table 4.** Recoveries (%) of diacetyl (DA) from broth supernatants of *Pediococcus* isolates

| Time/Isolate | LIV01 | FLE01 | FLE02 | INT01 | INT02 |
|---|---|---|---|---|---|
| 6h | 101.0±1.98 | 100.2±6.48 | 100.5±1.27 | 100.5±0.98 | 100.5±2.12 |
| 12h | 102.0±0.87 | 102.0±0.78 | 102.0±0.73 | 105.0±1.20 | 103.0±1.09 |
| 18h | 107.0±3.97 | 105.0±0.21 | 108.0±2.90 | 119.0±0.18 | 115.0±0.26 |
| 24h | 104.0±0.39 | 104.0±1.72 | 105.0±1.90 | 112.0±0.44 | 111.0±0.72 |
| 30h | 103.0±0.22 | 103.0±0.42 | 103.0±0.98 | 109.0±0.64 | 109.0±2.09 |
| 36h | 102.0±1.26 | 102.0±1.88 | 102.0±0.76 | 105.0±1.22 | 105.0±0.60 |
| 42h | 101.0±2.89 | 101.0±1.82 | 101.0±0.99 | 104.0±0.96 | 103.0±0.03 |
| 48h | 100.5±1.92 | 100.5±0.57 | 101.0±1.87 | 102.0±0.35 | 102.0±0.97 |

LIV01, FLE01, FLE02, INT01, and INT02 are as defined in Table 1

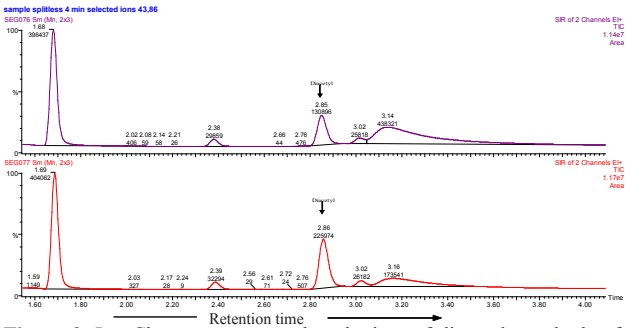


**Figure 2.** Ion Chromatograms and peak sizes of diacetyl standards of 0.75 mg/l (A) and 1.0 mg/l (B) obtained from GC. The DA peak is shown with an arrow

## Results

The isolation of the five *Pediococcus* strains, used in the present report, from Nigerian has been reported in a previous finding (Olaoye *et al*., 2008); they consisted of two strains of *Ped. acidilactici* and three of *Ped. pentosaceus*. In this study, the strains were screened for the production of antimicrobial agents, including lactic acid, acetic acid, hydrogen peroxide and diacetyl. Growth of *Ped. acidilactici* FLE01, and *Ped. acidilactici* FLE02 in the growth medium (MRS broth) within 48 h was better than that of the *Ped. pentosaceus* isolates (Figure 3), despite using the same cell numbers as the inoculum (~$10^6$ cfu/ml) for all isolates. Maximum counts of 2.5 x $10^9$ and 6.1 x $10^7$ cfu/ml were recorded for the strains of *Ped. acidilactici* and *Ped. pentosaceus* respectively.

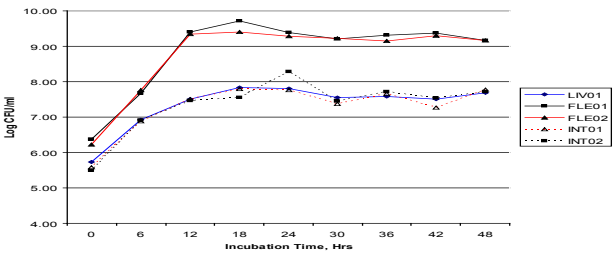


**Figure 3.** Growth of *Pediococcus* isolates (Log CFU/ml) in MRS broth during incubation at 30°C
LIV01, FLE01, FLE02, INT01, and INT02 are as defined in Table 1

The greater growth recorded for *Ped. acidilactici* strains FLE01 and FLE02 than those of *Ped. pentosaceus* did not correlate proportionally to their level of production of the antimicrobial agents (Table

5), as relatively lower concentrations were recorded for the two isolates. Lactic acid production increased with incubation time for all the isolates with the highest production of 23.37 and 28.02 (g/$10^7$ CFU) being recorded for *Ped. pentosaceus* INT02 and *Ped. pentosaceus* INT01 respectively within 42 h of incubation. The trend of acetic acid production was similar to that of lactic acid, with no detection noted of the two acids at 6 h of incubation. However, production of the acetic acid by the *Pediococcus* strains was lower than lactic acid throughout the incubation period. Diacetyl was generally produced in minimal amounts and concentration was observed to reach a maximum peak between 18 and 24 h by most of the strains, after which period a gradual decrease was noticed. The highest concentration of 57.89 µg/$10^7$ CFU was recorded for *Ped. pentosaceus* INT02. The result of pH measurement in the growth medium indicated a reduction to below 4.0 by all the *Pediococcus* strains within 24 h; this could be a useful factor in exertion of antagonism against spoilage and pathogenic organisms that may be associated with meat products.

The counts of the *Pediococcus* species measured during $H_2O_2$ production in phosphate buffer are presented in Table 5.6. The counts tended to remain approximately at the same level throughout the incubation period. However, a decline was observed after 24 h for most of the *Pediococcus* strains. Generally, estimation of hydrogen peroxide production by the *Pediococcus* strains was observed to be very low when compared to lactic and acetic acids. The antimicrobial agent was produced in very limited concentrations in phosphate buffer, with and without 55.5 mM glucose. No isolate showed production before 36 h and no production was observed for the strains LIV01, INT01 and INT02 at any time during incubation period. The highest production, 508.33 µg/$10^7$ CFU, was recorded for *Ped. acidilactici* FLE02 in the assay medium containing 55.5 mM glucose at 48 h. Generally, measurement of the agent was higher in growth medium containing 55.5 mM glucose than the one devoid of it.

**Table 5.** Results of pH, lactic acid, acetic acid and diacetyl measurements in MRS broth cultures of *Pediococcus* strains during growth

| Time (h) | LIV01 | FLE01 | FLE02 | INT01 | INT02 |
|---|---|---|---|---|---|
| ***pH*** | | | | | |
| 6 | 5.30 (±0.01) | 4.91 (±0.02) | 5.28 (±0.14) | 5.19 (±0.04) | 5.28 (±0.03) |
| 12 | 4.48 (±0.02) | 4.18 (±0.04) | 4.30 (±0.02) | 4.35 (±0.06) | 4.54 (±0.22) |
| 18 | 4.05 (±0.04) | 3.86 (±0.02) | 4.00 (±0.36) | 4.04 (±0.04) | 4.02 (±0.13) |
| 24 | 3.87 (±0.03) | 3.87 (±0.02) | 3.89 (±0.04) | 3.80 (±0.03) | 3.98 (±0.04) |
| 30 | 3.97 (±0.22) | 3.77 (±0.02) | 3.89 (±0.46) | 3.83 (±0.04) | 3.90 (±0.01) |
| 36 | 3.88 (±0.06) | 3.71 (±0.04) | 3.78 (±0.03) | 3.80 (±0.07) | 3.83 (±0.09) |
| 42 | 3.88 (±0.03) | 3.71 (±0.19) | 3.74 (±0.05) | 3.77 (±0.07) | 3.78 (±0.04) |
| 48 | 3.76 (±0.10) | 3.68 (±0.11) | 3.68 (±0.08) | 3.73 (±0.05) | 3.71 (±0.06) |
| ***Lactic acid*** (g/$10^7$ cfu) | | | | | |
| 6 | nd | nd | nd | nd | nd |
| 12 | 6.36 (±0.04) | 0.13 (±0.003) | 0.16 (±0.009) | 10.22 (±0.04) | 9.24 (±0.05) |
| 18 | 5.35 (±0.02) | 0.09 (±0.006) | 0.17 (±0.004) | 6.62 (±0.06) | 10.00 (±0.09) |
| 24 | 6.56 (±0.02) | 0.21 (±0.003) | 0.23 (±0.001) | 7.99 (±0.04) | 23.37 (±0.04) |
| 30 | 12.67 (±0.05) | 0.42 (±0.003) | 0.28 (±0.002) | 20.51 (±0.02) | 17.89 (±0.03) |
| 36 | 16.39 (±0.12) | 0.27 (±0.002) | 0.44 (±0.004) | 11.88 (±0.01) | 11.45 (±0.05) |
| 42 | 23.21 (±0.21) | 0.24 (±0.003) | 0.25 (±0.019) | 28.02 (±0.20) | 17.33 (±0.26) |
| 48 | 13.33 (±0.13) | 0.44 (±0.005) | 0.42 (±0.008) | 9.33 (±0.11) | 10.86 (±0.30) |
| ***Acetic acid*** (g/$10^7$ cfu) | | | | | |
| 6 | nd | nd | nd | nd | nd |
| 12 | 6.23 (±0.05) | 0.09 (±0.004) | 0.11 (±0.004) | 6.69 (±0.04) | 6.34 (±0.05) |
| 18 | 2.88 (±0.04) | 0.05 (±0.002) | 0.08 (±0.003) | 2.95 (±0.03) | 5.28 (±0.06) |
| 24 | 3.44 (±0.07) | 0.08 (±0.003) | 0.11 (±0.001) | 4.24 (±0.03) | 10.40 (±0.03) |
| 30 | 7.56 (±0.01) | 0.14 (±0.003) | 0.13 (±0.001) | 10.17 (±0.05) | 7.89 (±0.06) |
| 36 | 5.46 (±0.02) | 0.10 (±0.002) | 0.17 (±0.002) | 4.46 (±0.04) | 3.89 (±0.02) |
| 42 | 7.41 (±0.43) | 0.09 (±0.001) | 0.14 (±0.002) | 15.19 (±0.26) | 7.79 (±0.43) |
| 48 | 5.04 (±0.19) | 0.20 (±0.001) | 0.14 (±0.001) | 3.56 (±0.13) | 6.56 (±0.31) |
| ***Diacetyl*** (µg/$10^7$ cfu) | | | | | |
| 6 | 11.76 (±0.14) | 0.43 (±0.004) | 0.87 (±0.013) | 6.56 (±0.04) | 6.19 (±0.05) |
| 12 | 6.23 (±0.02) | 0.08 (±0.001) | 0.09 (±0.004) | 15.77 (±0.46) | 10.34 (±0.28) |
| 18 | 10.29 (±0.03) | 0.10 (±0.001) | 0.32 (±0.002) | 31.15 (±0.07) | 42.13 (±0.04) |
| 24 | 6.25 (±0.05) | 0.17 (±0.002) | 0.26 (±0.001) | 20.83 (±0.04) | 57.89 (±0.28) |
| 30 | 8.33 (±0.04) | 0.19 (±0.001) | 0.18 (±0.002) | 38.14 (±0.16) | 31.69 (±0.05) |
| 36 | 5.15 (±0.07) | 0.10 (±0.001) | 0.14 (±0.002) | 11.16 (±0.10) | 9.54 (±0.03) |
| 42 | 3.09 (±0.09) | 0.04 (±0.000) | 0.05 (±0.001) | 21.39 (±0.14) | 8.72 (±0.06) |
| 48 | 1.02 (±0.01) | 0.03 (±0.000) | 0.07 (±0.001) | 3.36 (±0.03) | 3.91 (±0.10) |

LIV01, FLE01, FLE02, INT01, and INT02 are as defined in Table 1; nd – not detected; cfu – colony forming unit
Figures are means of three replicates

**Table 6.** Colony forming unit (Log CFU/ml) of *Pediococcus* isolates during $H_2O_2$ production in phosphate buffer, with or without 55.5 mM glucose

| | Incubation Time (Hrs) | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 6 | 12 | | 18 | | 24 | | 30 | | 36 | | 42 | | 48 | | | |
| *Pediococcus* Isolate | | | NG | G | NG | G | NG | G | NG | G | NG | G | NG | G | NG | G | NG | G |
| LIV01 | 8.45 (±0.12) | 8.36 (±0.19) | 8.36 (±0.09) | 8.26 (±0.10) | 8.28 (±0.03) | 8.30 (±0.12) | 8.38 (±0.03) | 8.32 (±0.11) | 8.34 (±0.01) | 8.08 (±0.02) | 8.11 (±0.07) | 8.23 (±0.12) | 8.28 (±0.09) | 6.28 (±0.17) | 6.32 (±0.11) | 7.51 (±0.21) | 8.57 (±0.18) | |
| FLE01 | 9.30 (±0.22) | 9.57 (±0.02) | 9.51 (±0.09) | 9.43 (±0.37) | 9.20 (±0.02) | 9.34 (±0.03) | 9.30 (±0.00) | 8.99 (±0.19) | 9.00 (±0.10) | 8.79 (±0.12) | 8.81 (±0.02) | 7.54 (±1.02) | 7.53 (±0.20) | 6.18 (±0.02) | 6.52 (±0.08) | 6.15 (±0.10) | 6.23 (±0.12) | |
| FLE02 | 9.26 (±0.05) | 9.32 (±0.07) | 9.40 (±1.00) | 9.08 (±0.92) | 9.30 (±0.21) | 9.23 (±0.01) | 9.26 (±0.70) | 8.95 (±0.02) | 8.96 (±0.01) | 8.78 (±0.07) | 8.85 (±0.05) | 7.58 (±0.02) | 7.59 (±0.03) | 5.70 (±0.02) | 5.95 (±0.18) | 5.85 (±0.36) | 5.78 (±0.03) | |
| INT01 | 8.51 (±1.02) | 8.51 (±0.03) | 8.48 (±0.09) | 8.82 (±0.21) | 8.48 (±0.39) | 8.54 (±0.01) | 8.67 (±0.12) | 8.63 (±0.21) | 8.69 (±1.02) | 8.40 (±0.92) | 8.53 (±0.03) | 8.18 (±0.22) | 8.26 (±0.01) | 8.00 (±0.02) | 8.15 (±0.01) | 7.80 (±0.03) | 7.81 (±0.39) | |
| INT02 | 9.08 (±0.01) | 8.69 (±0.02) | 8.69 (±0.21) | 8.73 (±1.02) | 8.64 (±0.03) | 8.61 (±0.02) | 8.62 (±0.04) | 8.61 (±0.09) | 8.59 (±1.04) | 8.53 (±1.11) | 8.62 (±0.02) | 8.23 (±0.09) | 8.32 (±0.30) | 8.08 (±0.29) | 8.28 (±0.01) | 7.79 (±0.06) | 7.81 (±0.03) | |

NG= No glucose; G= 55.5 mM glucose
LIV01, FLE01, FLE02, INT01, and INT02 are as defined in Table 1

## Discussion

A modified HPLC-based method was used in the present study for the simultaneous separation and quantification of lactic acid and acetic acid production by the *Pediococcus* strains in MRS broth. Although there have been reports on the use of HPLC to separate the organic acids from other media (Fernandez-Garcia and McGregor, 1994; Zotou *et al*., 2004), the methods had to be modified before good separation of the acids could be obtained in this study. This could be due to differences in the media from which separation of the organic acids were to be effected. From the present findings, the retention time (RT) obtained by the modified method was lower than those reported by other research workers. In a method to measure lactic and acetic acids in wine, Zotou *et al*. (2004) reported RTs of 6 min (360 sec) and 6.5 min (390 sec) for the respective acids. Similarly, Fernandez-Garcia and McGregor (1994) obtained 12 (720 sec) and 14 (840 sec) when measuring the lactic and acetic acids respectively in yoghurt. The differences in the RT could also be probably be due to varying HPLC conditions used during assay and method of extraction of the acids (Isimer *et al*., 1991). The lower RT recorded in the current study would reduce the time required for analysis, thereby allowing an increased throughput of samples.

Although the strains of *Ped. acidilactici* appear to be better growth competitors than those of *Ped. pentosaceus*, the strains of the former were however poor producers of lactic acid, acetic acid and diacetyl. Hence, the antimicrobial activities of the latter strains could make them better potential starter cultures in preventing the growth of undesirable spoilage and pathogenic bacteria during food preservation processes (Leroy and De Vuyst, 2004; Ammor and Mayo, 2007). The early production of comparatively higher concentrations of lactic acid by *Ped. pentosaceus* INT02, compared to others during the incubation period, could serve as an important factor in competitive exclusion of unwanted organisms. Early production of antimicrobial agents, by starter cultures, has been reported to be of significance in food fermentation processes (Lucke, 2000; Hansen, 2002).

Furthermore, based on the performance of the *Pediococcus* strains in terms of DA production, it seemed *Ped. pentosaceus* INT02 could make a good protective culture. The strain produced a comparatively higher concentration of the antimicrobial than other strains. Production of relatively high DA concentration has been observed to contribute significantly to exertion of antagonism by *Pediococcus* against most unwanted organisms (Jay, 1982; Jyoti *et al*., 2003), hence the ability of strain *Ped. pentosaceus* INT02 in producing the antimicrobial could contribute to its potential use as a protective culture in meat preservation. However, from the result of DA production by the *Pediococcus* strains, the concentrations may not be sufficient to offer the required protection in food products. This is because a minimum concentration of 1 mg/l diacetyl has been demonstrated as required for effective inhibition against most of the spoilage organisms (Jay, 1982). Lanciotti *et al*. (2003) concluded that up to 300 mg/l is required to increase the lag phase when the antimicrobial activity of diacetyl was evaluated against *Escherichia coli*, *Listeria monocytogenes* and *Staphylococcus aureus*. Notably, the general low level of diacetyl production by the *Pediococcus* strains could be due to their homo-fermentative nature. Hetero-fermentative LAB have been known to produce the molecule in higher concentrations with a few exceptions, such as *Lactococcus lactis* (Hugenholtz *et al*., 2000; Joyti *et al*., 2003). The production of diacetyl in the early stage of incubation by the *Pediococcus* strains in this study is in support of Joyti *et al*. (2003), who observed maximum production of the antimicrobial between 15-20 h among the *Pediococcus* tested.

Production of high concentrations of hydrogen

peroxide, although antimicrobial, can contribute to loss in food qualities when present as it can interfere with the organoleptic properties of fermented meat products, by promoting rancidity and discoloration of the final product (Nordal and Slinde, 1980; Ammor and Mayo, 2007). However, it may be readily broken down by the catalase activity of haemoglobin, known to be naturally present in meat, when cultures are applied in the product (Nordal and Slinde, 1980; Noonpakdee *et al*., 2004; Ammor *et al*., 2005). In this study, the higher production of hydrogen peroxide observed in phosphate buffer supplemented with glucose than in those devoid of it could be attributed to higher metabolic activities of *Pediococcus* in the presence of the carbon source. Similar observations have been reported by other researchers (Villegas and Gilliland, 1998; Jaroni and Brashears, 2000) who assayed for the compound in sodium phosphate medium. The unsuitability of MRS medium for the assay of hydrogen peroxide production by LAB has been noted (Berthier, 1993; Rodriguez *et al*., 1997b). According to these researchers, there is breakdown of the compound in MRS and this has made it impossible to quantitatively determine its production in this growth medium.

Conclusively, the modified HPLC based method gave good separation of the lactic and acetic acids from MRS broth supernatants of the *Pediococcus* isolates. A short retention time generated by the method would promote high throughput for assaying of the organic acids, especially when a large volume of samples is to be analysed. The *Pediococcus* strains demonstrated production of the antimicrobial agent in varying concentrations. For strains showing promising potential as protective cultures, those with the ability to produce enhanced quantities of diacetyl and lactic acid should be favoured when selecting for use in food biopreservation, especially in meat products. This is due to their associated antimicrobial properties and these compounds do not usually present adverse effects on sensory qualities at concentrations commonly produced by LAB.

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