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Biosynthesis of Antimicrobial Compounds by Lactic Acid Bacteria and Its Use as Biopreservative in Pineapple Juice

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Abstract Lactic Acid Bacteria (LAB) are able to synthesize antimicrobial compounds which have both functional and technological properties in foods. This ability of LAB can be explored for use as bio-preservative in food. This study aims at identifying LAB isolated from fresh milk and employing it as natural preservative in extending the shelf life of Pineapple juice. Twenty strains of LAB were isolated from fresh milk and were identified as *L. fermentum, L. plantarum* and *L. lactis* respectively. *L. fermentum* produced the highest antimicrobial compound Lactic acid, diacetyl and hydrogen peroxide (1.8, 0.15, 0.03 g/l) respectively and the least was produced by *L*. lactis (1.2, 0.10, 0.02 g/l) respectively. Samples were pasteurized at 85°C for 15mins after which lactic cultures were introduced to the fresh pineapple juice singly and in combination. The shelf life and microbial load of the inoculated samples and control without the lactic cultures were monitored at room temperature for 7 days. LAB count in the inoculated samples increased from $3.1x10^3$ - $8.2x10^6$ cfu/ml from day 0 to 5 with no LAB growth noticed in the control. Yeast and Coliform Count increased after day 5 to ($5.1x10^4$ and $2.3x10^3$ cfu/ml respectively) but for control increased to $6.3x10^5$ and $5.2x10^4$ respectively. Aerobic plate count increased to $3.0x10^4$ cfu/ml after day 5 and $7.4x10^5$ for control. A decrease was observed in LAB count after day 5 to $3.2x10^5$cfu/ml. Lactic cultures extended the shelf life of Pineapple juice for 5 days after which there was deterioration monitored by increased count and decreased LAB activity. LAB exhibited a high antimicrobial effect on food borne contaminants. This ability of LAB can be employed as biopreservatives against food pathogens which also help to maintain and preserve the nutritive qualities of pineapple juice for an extended shelf life.

Keywords Lactic acid Bacteria, Shelf life, Antimicrobial Agent, Probiotics, Biopreservation, Microbial load

1. Introduction

Fresh and processed fruits consumption has continued to grow rapidly in recent times, mainly because of the need for a balanced diet, the health benefits, low calories in fruits and the superior flavor of the fresh fruits as compared to canned fruits (Mohammed, 2007, Ragaert *et al.,* 2004). One of the many popular fruit juice products is the pineapple (*Ananas comosus*) juice.

Today, pineapples are marketed as fresh and canned fruits in the United States and other nations and they are mostly used as tropical foods for many recipes including fruit salads, jams, juices and other products (Cho *et al.,* 2004). Pineapples have exceptional juiciness and a vibrant tropical flavor that balances the tastes of sweet and tart. The worldwide total pineapple production is between 16 to 19 million tons (Fernadens *et al*., 2008; FAO, 2009). The juice from pineapple is widely consumed by both adult and children.

Pineapple juice has a proximate composition of 81.2-86.2% moisture, 13-19% total soluble solid of which sucrose, glucose, and fructose are the main components, 0.4% fibre and rich source of vitamin C (Dull, 2000). Lipids and nitrogenous compounds constitute about 0.2%. The shelf life of the juice may be very short if it is not properly preserved.

The traditional method of preparation exposes the juice to microbial contamination through various means (Olubukola *et al.*, 2011). One of the possible causes of the short shelf life would be the presence of a high microbial population along the processing chain. Pineapple fruits can be contaminated in the field during harvesting, postharvest handling, and processing, shipping, marketing or in the home (Fernadens *et al*., 2008). Many microorganisms in particular acid loving bacteria such as some groups of LAB or acid tolerant bacteria such as *Staphylococcus* sp, *Bacillus* sp, *Pseudomonas* sp, *Micrococcus* sp, *Flavobacterium* sp etc; fungi such as *Aspergillus* sp, *Mucor* sp, *Fusarium* sp, *Penicillium* sp; and yeasts such as *Geotrichum* sp*, Candida* sp*, Saccharomyces sp, Schizosaccharomyces sp; Hanseniaspora sp; Pichia sp etc* can use this fruit as a substrate and cause spoilage producing off flavours, odours, discolouration of the product and if contaminating microorganisms are pathogens could cause illness

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(Simmonete et al., 2016).

However, an effective method of fruit preservation should retain the original characteristics of fruit as convenient as possible. The main methods of fruit preservation includes; Modified Atmosphere Storage (MAS), Proper washing with Potable water, Controlled Atmosphere Storage (CAS), use of preservatives, use of irradiation, use of heat, chilling and processing all of which extend the shelf life of fresh fruit produce (Dougheri *et al*., 2007).

Chemical preservatives such as sorbate and benzoate are the preservatives which have commonly been used in preserving fruit juice by extending its shelf life (Dougheri *et al*., 2007; Nwachukwu and Ezeigbo, 2013) but, these preservatives have been found to have adverse effects on the health of consumers. As a result of the health risks that are associated with consistent use of chemical preservatives, the search for a more appropriate, safe and hazard-free biopreservative technique has now become the current area of interest that is being explored in the fruit and beverage industries so as to minimize the risk effects among the demanding consumers.

However, antimicrobial compounds (lactic acid, diacetyl and hydrogen peroxide) produced by Lactic acid bacteria (LAB) have been proven to have great antimicrobial effect against fruit juice spoilage organisms without any adverse effect on the consumers (Mohammad, 2007). Hence, this study aims at identifying LAB isolated from fresh milk and employing it as natural biopreservative in extending the shelf life of pine apple juice.

2. Materials and Methods

Sample collection

Replicate samples of fresh milk were obtained from Fulani herdsmen at Osogbo, Osun State, Nigeria. These were collected in sterile universal bottles and kept at temperature of 4^{o}C before reaching the laboratory for microbial analysis. The samples were collected at different times and at different locations.

Preparation and sterilization of media

The media used in this experiment for the isolation of LAB are De Man Rogosa Sharpe (MRS) Agar, De Man Rogosa Sharpe broth, Nutrient Agar, MacConkey Agar and Yeast Extract Agar. The media were prepared following the standard laboratory methods as described by Cheesebrough (2003).

Isolation of LAB from sample

Samples were serially diluted and 1ml was taken from the sample and inoculated using pour plated method on De Man Rogosa and Sharpe (MRS) agar. The MRS agar plates were incubated for 48hrs at 35^{o}C in anaerobic jar and subcultured severally to obtain a pure culture. Twenty pure isolates were obtained and maintained on agar slant for further characterization and identification (Bromberg *et al.,* 2004, Oyeleke and Manga, 2008).

Characterization and identification of LAB isolates

The isolates were characterized based on colony morphology, cell morphology and biochemical tests (Cheesbrough, 2003; Oyeleke and Manga, 2008). The biochemical tests include Gram staining, catalase test, gas formation from glucose, dextran production from sucrose, starch hydrolysis etc. All strains were also tested for acid production from L- arabinose, D-xylose, galactose, D-fructose, Lactose, D-raffinose, mannitol and ducitol (Tserovska *et al*., 2002) and their identities confirmed using Bergey's manual of Systematic Bacteriology(Sneath *et al.*, 2009).

Inoculum preparation and standardization

The inoculum was prepared by aseptically inoculating a colony picked from each of the LAB streaked plates into a sterile 9ml MRS broth in test tubes using a sterile inoculating loop. The inoculated test tubes were incubated for 5 days and the antimicrobial compounds produced were checked for days 0, 3, and 5. The LAB inoculum was standardized by using 0.5 McFarland standards. McFarland standard was used as reference to adjust the turbidity of microbial suspensions so that the number of cells was equivalent to $3X10^{3}$ cfu/ ml (Khunajakr *et al.,* 2008).

Determination of Lactic Acid Production by the Isolates

Estimation of lactic acid produced was determined by titration of 25ml of broth cultures of the test organisms (24hr old) with 0.1N NaOH. 3 drops of phenolphthalein were added as indicator. NaOH was then added slowly to the sample until a pink colour appeared. Each ml of 0.1N NaOH is equivalent to 90.08mg of lactic acid as stated in A.O.A.C (2000).

$$\text{Titratable acidity of lactic acid} = \frac{\text{mlNaOH} \times \text{N NaOH} \times \text{M.E} \times 100}{\text{Volume of sample used}}$$

ml NaOH = Volume of NaOH used
N NaOH – Normality of NaOH
M.E = Equivalenat factor = 90.08mg

Determination of Diacetyl Production by the Isolates

Diacetyl produced by the isolates was estimated by measuring 25ml of the broth cultures of the test isolates (24hrs) into conical flasks and 7.5ml hydroxylamine solution was used for residual titration. The flasks were titrated with 0.1N HCl to a green-yellow end-point using bromophenol blue as indicator. The equivalent factor of HCl to diacetyl is 21.52mg (AOAC, 2000).

$$\text{Ak} = \frac{(b - s)\,(100 - e)}{w}$$

Ak = Percentage of diacetyl
b = Number of ml of 0.1N HCl consumed in titration of the sample
e = Equivalence factor = 21.52mg
w = Volume of sample
s = Number of ml of 0.1N HCl consumed in titration of

residue sample

Determination of Hydrogen Peroxide Production by the Isolates

About 20ml of diluted H_2SO_4 were added to 25ml of the broth cultures of the test organisms (24hrs). Titration was carried out with 0.1N $KMnO_4$. Each ml is equivalent to 1.70mg of H_2O_2 and declourization of the sample was regarded as the end point (AOAC, 2000).

$$H_2O_2 \text{ Concentration} = \frac{\text{ml}KMnO_4 \times NKMnO_4 \times \text{M.E} \times 100}{\text{Ml } H_2SO_4 \times \text{Volume of sample used}}$$

Ml $KMNO_4$ = Volume of acid used
$NKMnO_4$ = Normality of $KMnO_4$
Ml H_2SO_4 = Volume of H_2SO_4 added
M.E = Equivalent factor = 1.70mg

LAB selection for bio preservation of pineapple juice

The LAB isolates with high yield of antimicrobial compounds were selected for biopreservation of pineapple juice and these were *Lactobacillus fermentum* ML_6 and *Lactobacillus plantarum* ML_3.

Preparation and extraction of pineapple juice

Ripe and wholesome pineapples used were peeled, cut into pieces and blended with electric blender. The extract obtained was filtered to separate the juice from pineapple tissue. The pineapple juice was stored in a sterile container for further use (Odebunmi and Dosumu, 2003).

Pasteurization of the pineapple juice

The pine apple juice was pasteurized using High –Temperature-Short time method with water bath pasteurizer at 85^oC for 15minutes in sterile McCartney bottles (Odebunmi and Dosumu, 2003).

LAB selection for bio preservation of pineapple juice

The LAB isolates with high yields of antimicrobial compounds (lactic acid, diacetyl and hydrogen peroxide) were selected for bio preservation of pineapple juice and these were *Lactobacillus fermentum* ML_6 and *Lactobacillus plantarum* ML_3.

Biopreservative activity of LAB on pineapple juice.

One millilitre of standardized inoculums of *L. fermentum* ML_6 and *L. plantarum* ML_3 (combined lactic cultures) was aseptically pipetted into 10ml of the pasteurized pineapple juice inside McCartney bottles and one millilitre of standardized inoculum of *L. fermentum* ML6 and *L. plantarum* ML_3 (single lactic culture) was also pipetted into McCartney bottles containing 10ml of the juice samples differently. The sample bottles were stored at room temperature and monitored for a period of 7days.

Monitoring of the parameters of the fruit juice

(i) Physical appearance of the juice

The physical appearance based on colour of the pineapple juice was monitored for seven days according to the method of Mohammed *et al*. (2013).

(ii) Flavour determination

The flavour of the juice was monitored for seven days according to the method of Mohammed *et al*. (2013).

(iii) Microbial Count

The microbial colony count of the pine apple juice was taken for the seven days of storage and pour plate method was used. A tenfold serial dilution was carried out for both the single and combined cultures of LAB. One millilitre of the various samples were also serially diluted to 10 fold and Nutrient Agar, MacConkey Agar, Yeast Extract Agar and De man Rogosa Sharpe Agar (MRSA) were aseptically poured into the sterile Petri dishes (Cheesbrough, 2000). The Petri dishes were incubated aerobically at 35°C for 24hrs but for the MRS plates for 48hrs anaerobically. The total viable colonies were counted and recorded as colony forming unit per millilitre (cfu/ml) of the sample (Cheesbrough, 2003, Oyeleke and Manga, 2008).

3. Results

Twenty LAB isolates were obtained from the milk samples and were characterized. They were identified as *L. fermentum*, *L. plantarum* and *L. lactis* respectively. The isolate *L. fermentum* has the highest rate of occurrence of 60%, *L. plantarum* 20% and *L. lactis* 20%.

The pH of the lactic cultures was determined on 5 days of growth and the result is presented in Table 1. *L. fermentum* had the lowest pH, followed by *L. plantarum* and *L. lactis* (3.8, 4.1, 4.2) respectively.

Table 1. The pH of the lactic cultures on days 0-5

Isolates	pH Day 0	pH Day 3	pH Day 5
***L. fermentum* (ML_6)**	5.4	4.1	3.8
***L. plantarum* (ML_3)**	5.3	4.1	4.1
***L. lactis* (ML_2)**	5.3	4.1	4.2

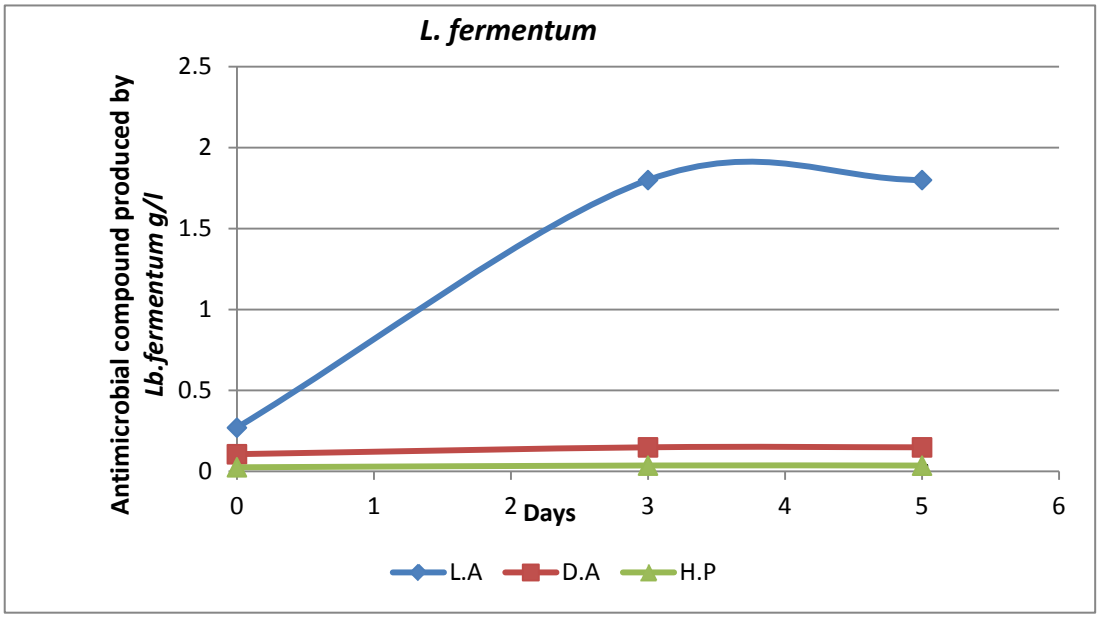
Antimicrobial compounds production by the LAB isolates were also investigated. This is represented in Figures 1, 2 and 3 respectively. The isolate *L. fermentum* produced the highest antimicrobial compounds of lactic acid, diacetyl and hydrogen peroxide (1.8, 0.149 and 0.0358g/l) respectively followed by *L. plantarum* (1.6, 0.1357, 0.0298g/l) and the least was produced by *L.lactis* (1.2, 0.1035, 0.043g/l) respectively.

The effect of combined cultures of *L. fermentum* and *L. plantarum* used as biopreservative on the microbial load of pineapple juice monitored for 7 days is shown on Figures 4 and the effect of single starter of *L. fermentum* used as biopreservative on the microbial load of pineapple juice monitored for seven days is shown in Figure 6. There was significant different between the microbial load of the samples between day 0-5 and after day 5.

The Microbial load of uninoculated pineapple juice without lactic cultures monitored for seven days is presented

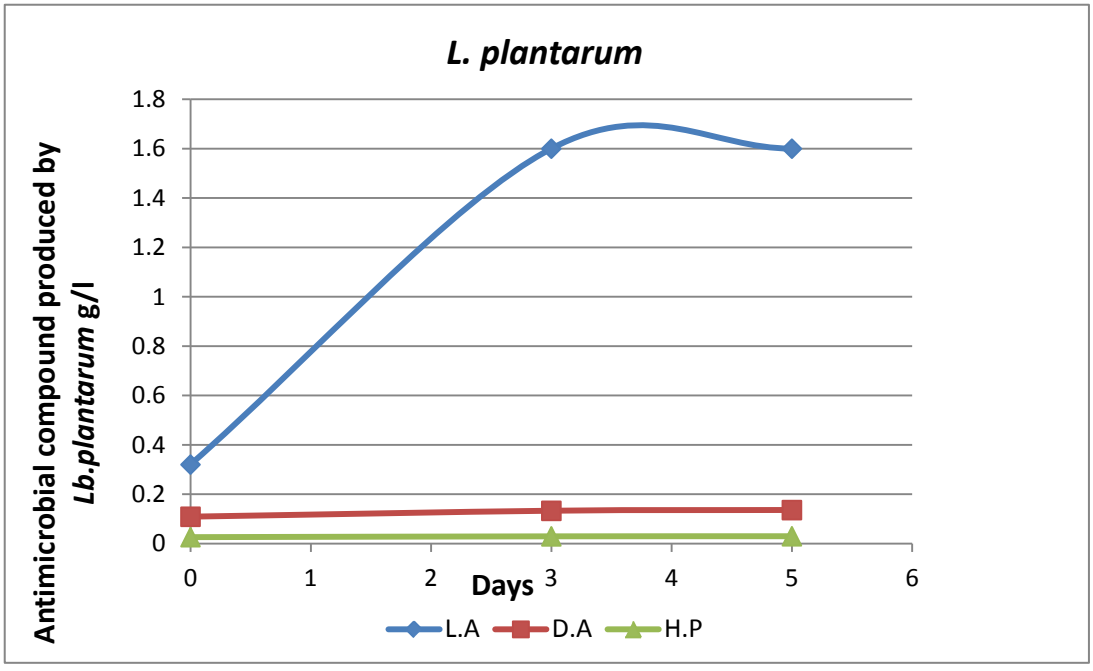
on Figure 5. There was a significant increase in the microbial load of the samples from day 2 -7 and the number of organisms increased on a daily basis because no preservative was added.

A creamy colour was observed in the appearance of the inoculated juice on day 0 to 5 which indicated retention of its texture but in the control, the colour was lightly brown after day 5. The flavour was also pleasant in the inoculated juice from day 0 to 5 but became unpleasant after day 5. In the control without the lactic cultures, the flavour became unpleasant after the first day and off-flavour developed in the juice. The microbial load also increased after day zero because no preservative was added.



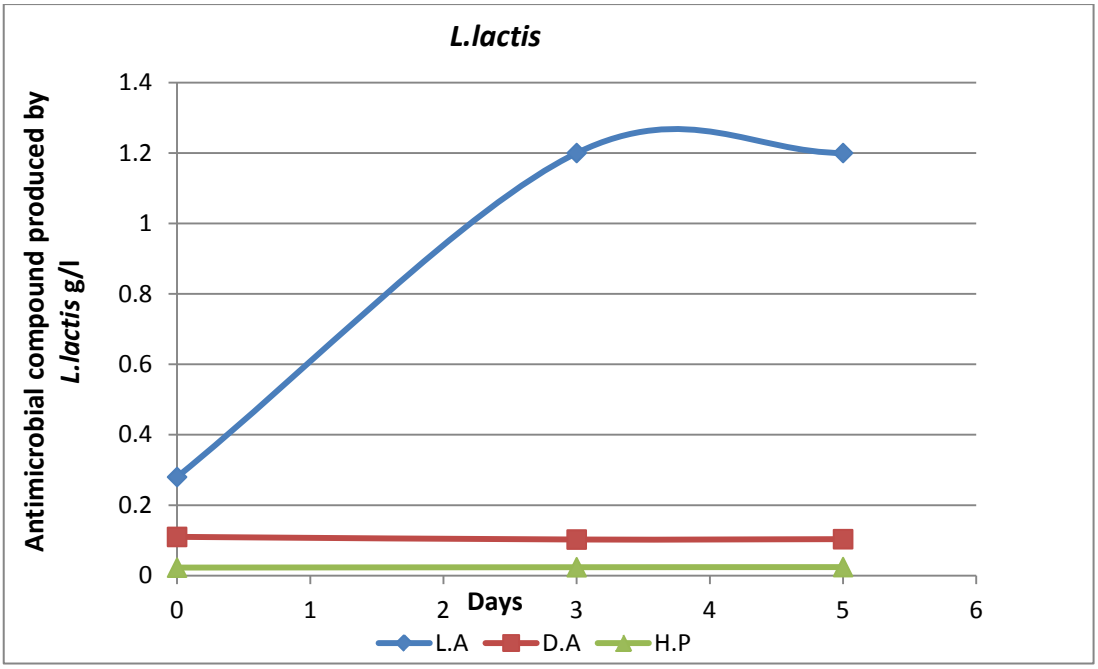
Key: L.A: Lactic acid, D.A: Diacetyl: H.P: Hydrogen peroxide

Figure 1. Antimicrobial compounds produced by *L. fermentum* monitored for 7 days



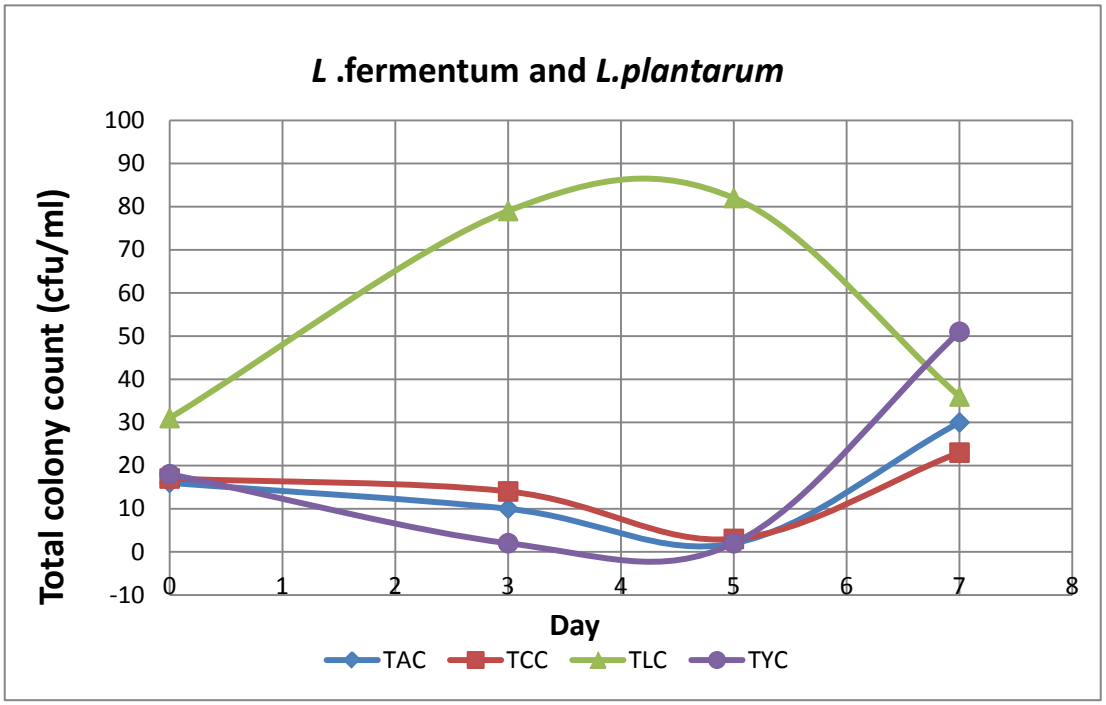
Key: L.A: Lactic Acid, D.A: Diacetyl, H.P: Hydrogen Peroxide

Figure 2. Antimicrobial compound produced by *L. plantarum* monitored for 7 days



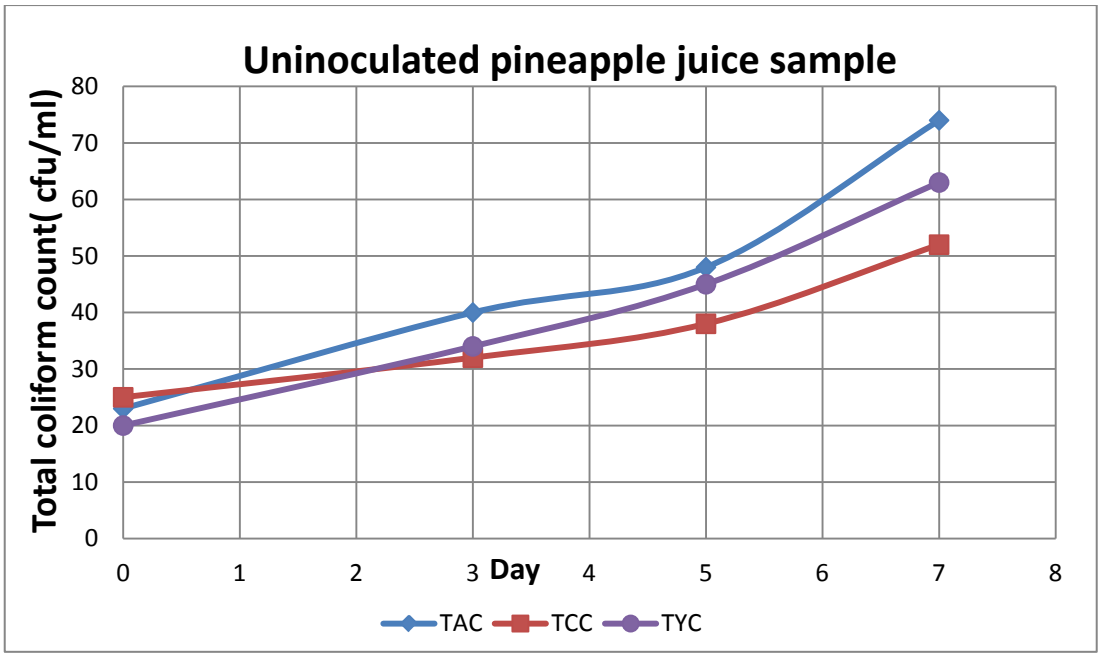
Keys: L.A: Lactic Acid, D.A: Diacetyl, H.P: Hydrogen Peroxide.

Figure 3. Antimicrobial compound production by *L. lactis* monitored for 7 days



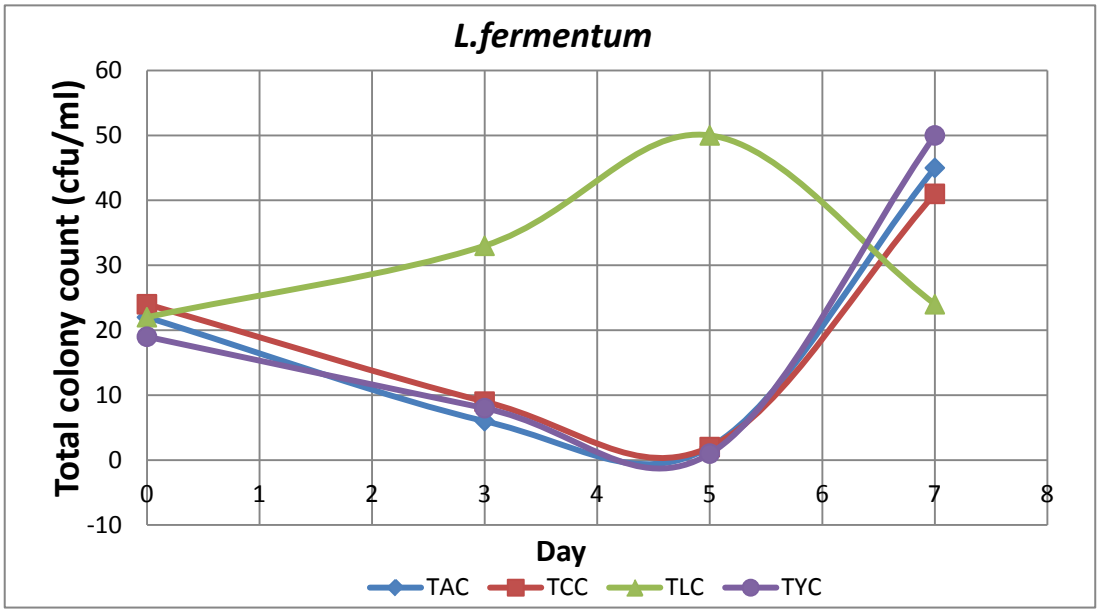
Keys: TAC; Total Aerobic Count, TCC; Total Coliform Count, TLC; Total LAB count, TYC; Total Yeast Count.

Figure 4. Effect of combined cultures of *L. fermentum* and *L. plantarum* on the microbial load of pineapple juice monitored for 7 days



Keys: TAC; Total Aerobic Count, TCC; Total Coliform Count, TYC; Total Yeast Count.

Figure 5. Microbial load of uninoculated pineapple juice without Lactic cultures monitored for seven days



Keys: TAC; Total Aerobic Count, TCC; Total Coliform Count, TYC; Total Yeast Count.

Figure 6. Effect of single starter of *L. fermentum* on the microbial load of pineapple juice monitored for seven days

The result of combined lactic culture in the pineapple juice sample from day 0 to 5 showed that there was increase in LAB count from $3.1x10^3$cfu/ml to $8.2x10^6$cfu/ml and a reduction in the total yeast count from $1.8x10^4$cfu/ml to $2.0x10^3$cfu/ml but the control increased to $6.3x10^5$ and $5.2x10^4$ respectively while total coliform count decreased in the same manner from $1.7x10^4$cfu/ml to $1.3x10^3$cfu/ml but the control increased from $2.5 x10^4$ to $3.8x10^5$ while the total aerobic plate count decreased from $1.6x10^4$cfu/ml to $2.0 x10^3$cfu/ml and the control increased from $2.3x10^4$ to $4.8x10^5$. However, an increase was observed in total yeast count after day 5 from $2.0 x 10^3$cfu/ml to $5.1x10^4$cfu/ml and the control increased to $6.3x10^5$. The total coliform count also increased from $1.3x10^3$cfu/ml to $2.3x 10^3$cfu/ml after day 5 and the control increased to $5.2x10^5$ while the total aerobic plate count increased from $2.3x10^3$cfu/ml to $3.0x10^4$ with the control increasing to $7.4x10^5$ and the LAB count decreased to $3.2x10^5$. The control for the single lactic culture was the same as that as that of the combined lactic culture.

4. Discussion

A total number of 20 strains of LAB were isolated from milk samples in this study. The LAB strains were characterized and identified. The identified LAB were grouped into two. The first group is the Lactobacilli group that was represented by two species, *L. fermentum* (60%) and *L. plantarum* (20%). The cocci group is the second group that was identified as *L. lactis* (20%). The identification by classification into groups was in accordance with the method used by Tserovska *et al*. (2002) and Morek (2011). The lower number of lactic acid cocci is probably due to their inability to compete with lactic acid bacilli in mixed cultures (Togo *et al*., 2002). The morphological, biochemical and physiological characterization of the isolates revealed that the isolates that produced lactic acid in abundance belong to the *Lactobacilli* group which are *L. fermentum* and *L. plantarum*.

The variation of acidification was monitored for all the isolates. The decrease in the pH of lactic cultures after 48hr showed the lactic acid production efficiency of the isolates. Lactic acid bacteria are known to produce antimicrobial substances mainly in the form of organic acids and other metabolites (Tserovska *et al*., 2002; Obadina *et al*., 2006).

The LAB isolated from milk in this study produced different antimicrobial compounds and the quantity varies with time. The increase in the production of lactic acid with time has been attributed to lower pH which permits the growth of LAB. The LAB strain of *L. fermentum* isolated from the milk sample has the highest production of antimicrobial compounds on day 5. The production of antimicrobial compounds by these LAB strains confers the potential ability on them to extend the shelf life of fruit juice and reduce the microbial load.

Antimicrobial compounds can be applied to foods either as purified chemical agents, or as viable cultures in the case of fermented products (O'Sullivan *et al*., 2002). The inoculation of lactic cultures of *L. fermentum* ML6 and *L. plantarum* ML3 into pineapple juice revealed that pH, storage temperature and microbial load played significant roles in shelf life determination. This agrees with the report of FSAI (2011) that the shelf life of many food products is dependent on storage temperature and microbial load. At room storage temperature (23±10°C), the pineapple juice was preserved and this is probably due to the presence of lactic cultures which grows optimally at 30±5°C and produce metabolites like lactic acid, diacetyl, hydrogen peroxide and bacteriocin which probably inhibited the growth of spoilage and pathogenic microorganisms in the pineapple juice under study.

This is similar to the findings of Ogunbanwo *et al*. (2003) and FSAI (2011) who reported that LAB grows optimally at 30-37°C and produce metabolites like bacteriocin which probably inhibits the growth of spoilage and pathogenic microorganisms such as *S. aureus* and other microflora in the pineapple juice. Tserovska *et al*. (2002) also reported that (LAB) grow optimally at pH 5.8 to 6.5 and produce metabolites like lactic acid and bacteriocin (bio preservative) which are active against food borne pathogens. Similar observations were made in this study where lactic cultures inoculated into pineapple juice grew maximally and produced high amount of antimicrobial compounds at pH 3.8 and 4.0.

The lactic cultures (*L. fermentum* ML6 and *L. plantarum* ML3) inoculated into pineapple juice proved effective in reducing the microbial load, and hence spoilage of the product and extended the shelf life of pineapple juice at room temperature for 5 days. The shelf life extension of pine apple juice could be as a result of increased antimicrobial activity of the lactic cultures and hence biopreservation. This is in correlation with the report of O'Sullivan *et al*. (2002) and Tserovska *et al*. (2002) who opined that; as an alternative to using antimicrobial compounds for bio preservation of foods, direct introduction of live antimicrobial-producing culture of LAB as a protective starter could help in achieving favourable results in some food systems. This contribution is due to competition for nutrients and the presence of inhibitor agents produced, including organic acids, hydrogen peroxide, and bacteriocins.

The antimicrobial compounds produced by LAB obtained from milk may be used to solve the problem of growth of contaminating microorganisms during processing in the food industry. The use of lactic cultures with biopreservative activity could improve the quality of food and increase its safety by inhibiting the food-borne pathogens and spoilage microorganisms.

5. Conclusions

The growth of pathogenic and spoilage microorganisms in pineapple juice under study were inhibited by *L. fermentum* and *L. plantarum*. LAB are very important in that their presence in food can enhance the extension of the shelf life of the food products and reduce the microbial load. Therefore, the presence of antimicrobial compounds producing LAB in juices and fermented foods can enhance the safety and shelf life extension of the food products and will also serve as alternative to chemical preservatives or additives in food preservation.

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