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EVALUATION OF ANTIBACTERIAL AND SANITIZING ACTIVITY OF *Lactobacillus plantarum* AGAINST *Staphylococcus aureus* IN STORED *Citrus lanatus* (MELON) DRINK

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Abstract
The main study objective was to isolate and characterize some lactic acid bacteria from some fermented foods and evaluate their ability to produce antagonistic effect against some food-borne pathogens as biopreservatives in the form of protective cultures or their metabolites. Isolation and Identification of *Lactobacillus* species from some fermented drinks was carried out on six different food types in triplicates following standard procedures. Following Isolation and Identification by biochemical and molecular methods, the antimicrobial activity of the *Lactobacillus* isolates in three different densities (1.5×10^8 cfu/mL, 2.1×10^8 cfu/mL and 3.0×10^8 cfu/ml) were evaluated against *Escherichia coli*, *Salmonella typhi*, *Salmonella typhimurium* and *Staphylococcus aureus* isolated from some foods. Sanitizing activity of *L.plantarum* against *S.aureus* in melon drink was evaluated at the time intervals of 0, 24 and 48 hours respectively. Results of Isolation and Identification revealed the isolate to be *L. plantarum*. Antibacterial activity of different densities of *Lactobacillus* showed that for 1.5×10^8 cfu/ml and 2.1×10^8 cfu/ml densities, the highest zone of inhibition produced were 17.00 ± 0.57 and 24.66 ± 0.88 against *S. typhi* and the least (6.66 ± 0.66) against *E. coli*. For 3.0×10^8 cfu/ml density, the highest zone of inhibition was 35.33 ± 0.88 against *S. typhi* and the least was 9.66 ± 1.66 against *E. coli*. Statistical analysis revealed significant difference ($P<0.05$) in the antimicrobial activity of three different densities of *Lactobacillus* against the tested bacterial isolates. Results of sanitizing activity of *L.plantarum* against *S.aureus* revealed 3.52 Log_{10}cfu/ml reduction after 24hours of storage. Conclusively, *Lactobacillus plantarum* could be employed as a biopreservative in stored melon drink.
Keywords: Fermentation, *Lactobacillus,* Melon, *Kunun Zaki,* Indigenous

INTRODUCTION

Developing countries like Nigeria require food processing technologies that are appropriate, suitable for tropical regions and affordable to rural and urban economies. Fermentation techniques are one of such technologies that have been developed indigenously for a wide range of food products (Pal *et al.,* 2005).The primary benefit of fermentation is the conversion of sugars and other carbohydrates to usable end products. The traditional fermentation of foods serves several functions, which includes: enhancement of diet through development of flavour, aroma, and texture in food substrates, preservation and shelf-life extension through lactic acid, alcohol, acetic acid and alkaline fermentation, enhancement of food quality with protein, essential amino acids, essential fatty acids and vitamins, improving digestibility and nutrient availability, detoxification of anti-nutrient through food fermentation processes, and a decrease in cooking time and fuel requirement (Evans *et al.,* 2013).

Lactic Acid Bacteria (LAB) to which *Lactobacillus* species belong are of particular interest to the food industry, since these bacteria have generally been regarded as safe (GRAS status). Their growth lowers both the carbohydrate content of the food that they ferment as well as the pH due to lactic acid production. The pH may drop to as low as 4.0, enough to inhibit the growth of most other microorganisms including the most common human pathogens, thus allowing these foods prolonged shelf life. These bacteria also exert a strong antagonistic effect against many microorganisms, including food spoilage organisms and pathogens (Jagadeeswari*et al.,* 2010) owing to the production of some primary metabolites and antimicrobial compounds like bacteriocins. Bukar and Nainna (2017) reported on the *in vitro* antibacterial activity of *L. salivarius* and *L. oris* against *E. coli* and *S. typhi* with zones of inhibition diameter range between 9.66 ± 0.66mm and 35.33 ± 0.88mm.

The antibacterial activity was associated with the production of antimicrobials such as bacteriocins and the acidic pH of the medium (Pal *et al.,*2005).

Lactobacillus species have a long history of application in fermented foods because of their beneficial influence on nutritional, organoleptic, and shelf-life characteristics (Leroy and Vuyet, 2004). It is in view of their importance in shelf life extension of food (Evans *et al.,* 2013), that this research was undertaken to evaluate the sanitizing activity of *L. plantarum* against *S. aureus* in stored melon drink.

MATERIALS AND METHODS

Collection of samples

Three (3) samples of "*Kunun zaki*" were collected aseptically from several vicinities within Kano metropolis, directly from sellers. Analysis of all food samples was carried out immediately on arrival at the laboratory.

Isolation of *Lactobacillus* specie

The samples of fermented foods were each inoculated into MRS Agar plates and incubated at 37°C for 48h. Well isolated colonies were picked and transferred to new MRS agar plates by streaking. Colonies showing typical characteristics of *Lactobacillus* species on agar surface were picked up and transferred onto MRS broth and Nutrient Agar for further enrichment and purification. The pure cultures were then subjected to identification (Cheesbrough, 2002).

Identification of *Lactobacillus* specie

Gram staining

A heat-fixed bacterial smear on a slide was stained with Crystal Violet for 1 minute and rinsed with water. It was then treated with Lugols Iodine for a minute, then rinsed with water and then treated with acetone which was rinsed immediately with water. The smear was then counter stained for 30 seconds with Safranin and then rinsed with water afterwards, left to air dry and examined under the microscope (Cheesebrough, 2002).

Catalase test

A loopful of bacterial culture was taken and mixed with 3% H_2O_2 solution on a clean microscopic slide and the presence of bubble was observed (Cheesebrough, 2002).

Endospore test

Bacterial smear was made on microscopic slide under aseptic conditions and heat fixed. The slide was placed over steaming water bath and Malachite green (primary stain) was applied for 5 minutes. The slide was then removed from the water bath and rinsed with water. Then the slide was flooded with Safranin for 20seconds and rinsed with water. The slides were blotted dry and observed under the microscope (Goyal *et al.,* 2012).

Sugar fermentation tests

The selected strain was further confirmed for production of acids from carbohydrates and related compounds by use of the API 50CHL system (BIOMÉRIEUX SA, France). Identification procedures were conducted in accordance with manufacturer's instructions. Portions of growth of the isolate was aseptically transferred from a freshly inoculated stock culture using a swab to an ampoule of API 50 CHL basal medium and then emulsified to give a final turbidity equivalent to McFarland standard No.2. Each tube of the API 50 CHL strip was inoculated with the bacterial suspension using a sterile pipette. The strip was placed in the incubation tray with honeycombed wells each filled with demineralized water according to the instructions of the manufacturer. The tray with the strips in it was covered loosely with a lid, and incubated at 37°C for 72hours. Reactions were visually examined after 24, 48 and 72hours and determined to be positive or negative based on colour change in the tube caused by production of acid and detected by the pH indicator present in the medium. The results, which form biochemical profiles, were identified using sugar fermentation patterns from previous studies **(**Azadnia and Khan, 2009; Khedid *et al.,* 2009; Asmahan, 2011**)**.

Source of food borne isolates

Food borne isolates used for bioassay are *Staphylococcus aureus, Escherichia coli*, *Salmonella typhimurium* and *Salmonella typhi* and were sourced from stale foods, which included melon and *zobo* drinks. Foodborne organisms were isolated on selective media and confirmed by Gram staining and Biochemical tests according to methods of Cheesebrough (2002).

Standardization of the inoculum

All inoculums were standardized using overnight cultures of bacterial isolates. Standardized culture of *L. plantarum* were prepared in three different densities 0.5, 0.7and 1.0 Mcfarland for both bioassay and sanitizing activity, while 0.5 Mcfarland was prepared for the food-borne isolates following standard procedures of Cheesebrough (2002).

Bioassay

Lactobacillus plantarum was grown in MRS broth at 37^0C for 48hours. Cells were then separated by centrifugation at 5000 rpm for 10 min. About 6 mm diameter wells was made on pre inoculated Mueller Hinton agar media and each well was filled with 100 µl of culture supernatant of the isolate following standardization to 0.5 McFarland standard (MFS).

Inhibitory activity was observed following incubation at 37^0C for 24 hours. Inhibition zones around the wells were observed by clear zones extending laterally from the border of the putative isolates, noted and recorded in mm diameter (Toba *et al.*, 1991; Ogunshe*et al.*, 2007; Goyal*et al.*, 2012). It was presumed that the greater the diameter of the Zone of Inhibition (ZOI), the greater the antimicrobial activity of the isolate.

Production and sterilization of Melon drink

This was prepared by aseptically blending 100g of melon with a blender and adding 1 litre (1L) of water (Bukar, 2013). The drink was then sieved and sterilized using a membrane filter (0.22 µm).

Treatments of Melon drink with *L. plantarum* and *S. aureus*

A set of four sterile 100 mL capacity sterilized bottles were filled with 50mL each of melon drink. Four (4) different treatments were carried out as follows:

A- Melon drink with 1.5×10^8 cfu/ml *S. aureus* $+1.5 \times 10^8$ cfu/mL *L. plantarum*

B- Melon drink with 1.5×10^8 cfu/ml *S.aureus* $+2.1 \times 10^8$cfu/mL *L. plantarum*

C- Melon drink with 1.5×10^8 cfu/ml *S.aureus* $+3.0 \times 10^8$cfu/mL *L. plantarum*

D- Melon drink with 1.5×10^8 cfu/ml *S. aureus* only (negative control)

Treatments were stored for 48hours. Treatments were carried out in triplicate. Mean values were recorded.

Evaluation of sanitizing effect of *L. plantarum* against *S. aureus*

Enumeration of *L. plantarum* and *S. aureus* at 0, 24 and 48hours was carried out by Pour plate technique (FAO, 1979**)**. This was carried out for all the three densities of the samples, where each was serially diluted by introducing 1 ml into 9 ml of sterile buffered peptone water in a test tube, which was labeled as 10^{-1}. One milliliter (1 ml) from the 10^{-1} was added to 9ml of sterile buffered peptone water and serially diluted to four other test tubes labeled (10^{-2} - 10^{-5}). The procedure was carried out for each of the samples. One milliliter (1 ml) aliquot of each dilution was pippeted and added to appropriately labeled sterile duplicate petri dishes. Warm MRS agar (for *L. plantarum*) and Mannitol Salt Agar (MSA) (for *S. aureus*) were added respectively and swirled, allowed to gel and then incubated at 37¤C for 24 hours. Discreet colonies on the plate labeled with 10^{-3} dilution were counted and multiplied by the inverse of the dilution factor (Cheesbrough, 2002).

Measurement of pH and temperature of stored melon drink

The pH and temperature of the stored treated melon drink were measured by using a Jenway pH meter and a thermometer after aseptically dispensing 5ml of each sample into clean sterile beakers respectively (Harrigan, 1998).

Statistical Analysis

Means of data generated for bioassay were statistically analyzed using one way ANOVA at 5% probability level operated using software Excel, developed my Microsoft©.

RESULTS AND DISCUSSION

Lactobacilli were isolated from a total of 3 samples of *Kunun zaki*. Colonies ranged between large and small, creamy to whitish in color, and circular in shape. Microscopy revealed Gram positive rods and cocci shaped bacteria. The Gram positive rods were then subjected to further examination. Endospore test showed that the isolate is a non-spore former. Catalase test was also negative. Morphological and sugar fermentation results of the isolate is presented in Table 1. Earlier studies conducted by Ogunshe *et al*. (2007) and Olanrewaju (2007) showed that this organism was isolated from a similar source.

Table 2 shows the result for the antimicrobial activity of the isolates in terms of diameter of the zone of inhibition (ZOI). The result demonstrated that *L. plantarum* had the highest antimicrobial activity against *S. typhi* (24.66 ± 0.88 and 35.33 ± 0.88), *S. typhimurium* (20.33 ± 0.88 and 34.33 ± 2.3) and *S. aureus* (16.33 ± 0.88 and 24.33 ± 0.33) at densities of 2.1×10^8 cfu/mL and 3.0×10^8 cfu/mL. The result also revealed a dose – dependent antibacterial activity with lower zones recorded at 1.5×10^8 cfu/mL and highest recorded at 3.0×10^8 cfu/mL of the isolate. Bioassay results yielded different antimicrobial spectra for all the isolates and the different densities tested. Similarly, this difference was also observed (Khalil *et al*., 2009; Yang *et al*., 2012), where *L. plantarum* was used against *S. aureus* and *S. typhimurium* with inhibitory activity more against *S. aureus* than *S. typhimurium*. In this study, there was an increase in microbial activity going by the overall result as the microbial density increased. The relatively low density of *Lactobacilli* isolates might have contributed to less inhibitory effect against some of the pathogens. In a study conducted by Kumar and Murugalatha (2012) where *Lactobacillus* was isolated from cow milk and its antimicrobial activity against some pathogens using culture supernatant evaluated. The organism showed strong activity against S. *aureus,* however, S. *typhi* was resistant to it.

The inhibitory spectrum against variety of Gram positive and negative pathogens was widely varied. It was observed that *Lactobacillus* had an inhibitory effect on *S. aureus* and some other Gram positive bacteria, while none of them affected *S. typhi* the other Gram negative pathogens used (Weber and Broich 1986; Tortorello*et al.*, 1991).

Table 1: Isolation and Biochemical Identification of *Lactobacillus* specie from some fermented foods

Cell morphology	Bacilli
Grams reaction	+
Catalase	-
Spore test	-
Sugar fermentation	-
Glycerol	-
Erythriol	-
D-arabinose	-
L-arabinose	+
Ribose	+
D-xylose	+
L-xylose	-
Adonitol	-
ß-metil-D-xyloside	-
Galactose	+
D-glucose	+
D-fructose	+
D-mannose	+
L-sorbose	-
Rhamnose	-
Dulcitol	-
Inositol	-
Mannitol	+
Sorbitol	+
α-methyl-D-mannoside	-
α-methyl-D-glucoside	-
N-acetyl-glucosamime	+
Amigdalin	+
Arbutin	+
Esculin	+
Salicin	+
Cellobiose	+
Maltose	+
Lactose	+
Melibiose	+
Saccharose	+
Trehalose	+
Inulin	-
Melezitose	+
D-raffinose	+
Amidon	-
Glycogen	-
Xylitol	-
ß-gentiobiose	+
D-turanose	-
D-lyxose	-
D-tagarose	+
D-fuccose	-
L-fuccose	-
D-arabitol	-
L-arabitol	-
Gluconate	+
2-keto-gluconate	-
5-keto-gluconate	-
Inference	*L. plantarum*

Key: K= Kunun zaki, += Positive reaction, - = negative reaction

Table 2: Antibacterial activity of different densities of *L. plantarum* showing diameter of inhibition (mm)

Isolate density (cfu/mL)	Zone of inhibition diameter (mm)				
	E. coli	***S. typhi***	***S. typhimurium***	***S. aureus***	**Statistics**
1.5×10^8	6.00 ± 0.00	17.00 ± 0.57	6.66 ± 0.00	12.66 ± 0.66	P <0.05
2.1×10^8	6.66 ± 0.66	24.66 ± 0.88	20.33 ± 0.88	16.33 ± 0.88	P <0.05
3.0×10^8	9.66 ± 0.66	35.33 ± 0.88	34.33 ± 2.33	24.33 ± 0.33	P <0.05

Values are mean ± standard error
Values are means of triplicate determinations

Figure 1 shows the result of mean staphylococcal count only, stored in melon drink for 48hrs. At 0hrs, log count was $3.52Log_{10}$cfu/ml, then to $6.4Log_{10}$cfu/ml at 24hrs and later increased to $6.79Log_{10}$cfu/ml at 48hrs. The pH at 0hrs was 7.15, then dropped to 6.86 at 24hrs and later 6.32 at 48hrs. The temperature was 31¤C at 0hours, 30¤C at 24hours and 33¤C at 48hours.

Figure 2 shows the mean staphylococcal and *L. plantarum* (1.5×10^8 cfu/mL) counts in melon drink stored for 48 hours. At 0hrs, staphylococcal log count was $3.52Log_{10}$cfu/ml and that of *L. plantarum* was $3.30Log_{10}$cfu/ml, while the pH was 6.0, however, after 24hrs, there was a change in pH to 5.9 and staphylococcal log count to 0 as *L. plantarum* count increased to $6.39Log_{10}$cfu/ml. At 48hrs, *L. plantarum* log count increased to $6.81Log_{10}$cfu/mL whereas staphylococcal log count remained 0. The temperature, was 31¤C at 0hours, 30¤C at 24hours and 33¤C at 48hours.

Figure 3 presents the mean staphylococcal and *L. plantarum* (2.1×10^8 cfu/mL) log count in melon drink stored for 48 hours. The Staphylococcal log count was$3.52Log_{10}$cfu/mL at 0hr and then 0 at both 24 and 48 hours even as that of *L. plantarum* was $6.32Log_{10}$cfu/mL, then increased to $6.51Log_{10}$cfu/ml and $6.93Log_{10}$cfu/ml at 0, 24 and 48hours. The pH slightly dropped from 6 to 5.9 and later 5.78. The temperature, was 31¤C at 0hours, 30°C at 24hours and 33 ° C at 48hours.

Figure 4 revealed the mean staphylococcal and *L. plantarum* (3.0×10^8 cfu/ml) count in melon drink stored for 48 hours. At 0hrs, *L. plantarum* count was $6.50Log_{10}$cfu/ml while staphylococcal count was $3.52Log_{10}$cfu/ml. At 24hrs, the staphylococcal count was 0 and that of *L. plantarum* slightly increased to $6.68Log_{10}$cfu/ml and later at 48 hours dropped to $4.62Log_{10}$cfu/mL. There was also a slight drop in pH by 0.05 and a slight increase in temperature by 2¤C. The temperature was 31¤C at 0hours, 30¤C at 24hours and 33¤C at 48hours. Generally, it should be observed that *Staphlococcal* count dropped from $3.52Log_{10}$cfu/mL to 0 in all densities. This could be attributed to earlier literature reported by Gilliland and Speck (1977) that, *Lactobacilli* showed stronger antibacterial properties against gram-positive bacteria than gram-negative bacteria in all the three different densities where LAB count kept increasing at 24 and 48 hours except in 3.0×10^8 cfu/mL treatment where it dropped. Here, the sanitizing effect of *L. plantarum* on *S. aureus* cannot be linked to a single factor, even though similar studies indicate that LAB strains were inoculated onto onions and the isolates inhibited the growth of the test pathogen (Yang *et al.*, 2012). The same was reported by Sharpe (2009) where LAB were inoculated into fresh cut salad consequently reducing *Pseudomonas* specie, yeasts and total coliforms. It is very interesting to know that LAB only reduced the counts but did not completely eradicate the pathogens as in the case of this study, however, Vescovo *et al*. (1996) reported that LAB effectively controlled the growth of undesirable bacteria in ready to use vegetables and Obadina *et al*. (2007) also demonstrated the complete eradication of *E. coli*, *S. aureus* and *S. typhi* in fermented cassava by *L. plantarum* after 60 hours and 92 hours respectively.

In this study, *L. plantarum* count kept on increasing, which might be as a result of difference in nutritional composition and presence of enzymes probably absent in melon drink. Furthermore, in 3.0×10^8 cfu/mL treatment, the count reduced to $4.62Log_{10}$cfu/mL, which could be due to overcrowding of the organisms, bringing about decline in the availability of nutrients, accumulation of waste matter and death. The increase in count could be as a result of differences in strains of the isolates, nutritional composition of the foods, enzymes, storage and environmental conditions.

The successful eradication of *S. aureus* by *L. plantarum* could be attributed to a combination of primary and secondary metabolites even though the production of bacteriocin is highest at the end of the exponential and early stationary phase (Daba *et al*., 1993; Thomas *et al*., 2000) and their degree of adsorption is pH dependent, with a maximum at about pH 6.0 and a minimum at or below pH 2.0 and at temperature 30¤C. Bacteriocin production is strongly dependent on pH, nutrients source and temperature as claimed by Todorov and Dicks (2004).

The inhibitory action of LAB can also be due to accumulation of primary metabolites such as lactic and acetic acids, ethanol and carbondioxide (Kazemipoor *et al*., 2012). Additionally, LAB are also capable of producing antimicrobial compounds such as formic acid, benzoic acid, hydrogen peroxide, diacetyl, acetoin and bacteriocins (Yateem, 2008). Finally, this implies that the selective use of *L. plantarum* may improve the microbiological quality of such foods by providing a reasonable assurance of the control of *Staphylococcus aureus*, with a proper sanitation procedure, good processing method combined with the addition of *L. plantarum* antimicrobial metabolites. Hence, *L. plantarum* could be used to improve the safety of traditional fermented foods, where *Lactobacillus* commonly occurs.

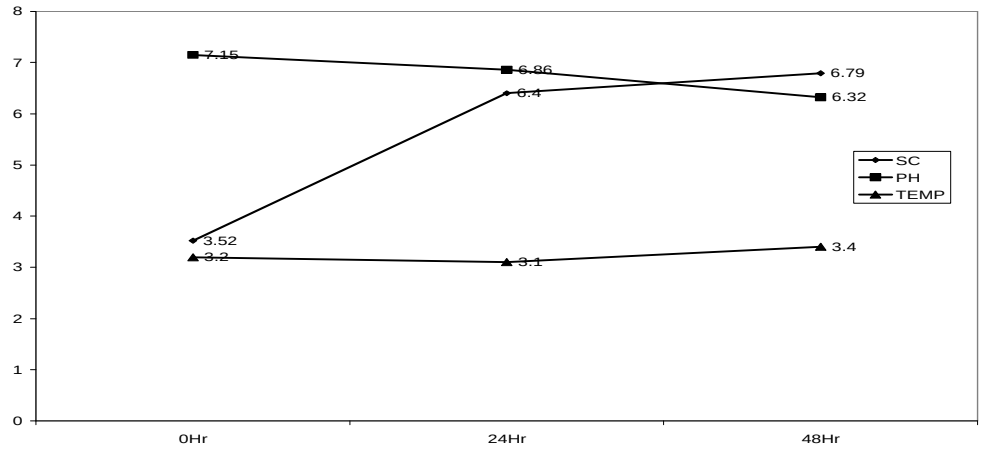

Figure 1: Mean staphylococcal counts in melon drink uninoculated with *L. plantarum* stored for 48 hours at ambient Temperature.
Key: SC= Staphylococcal count

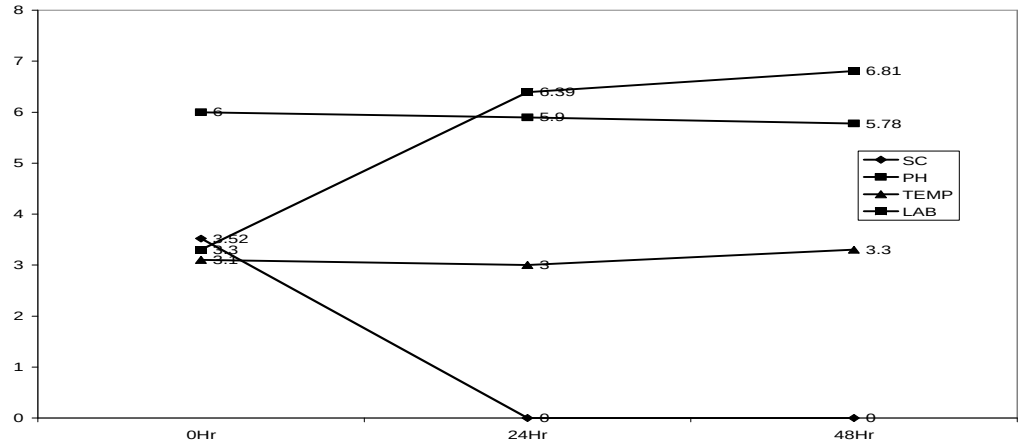

Figure 2: Mean staphylococcal counts of melon drink inoculated with *L. plantarum* (1.5×10^8 cfu/ml) stored for 48 hours at ambient Temperature.
Key: SC= Staphylococcal count, LAB= *L. plantarum* count

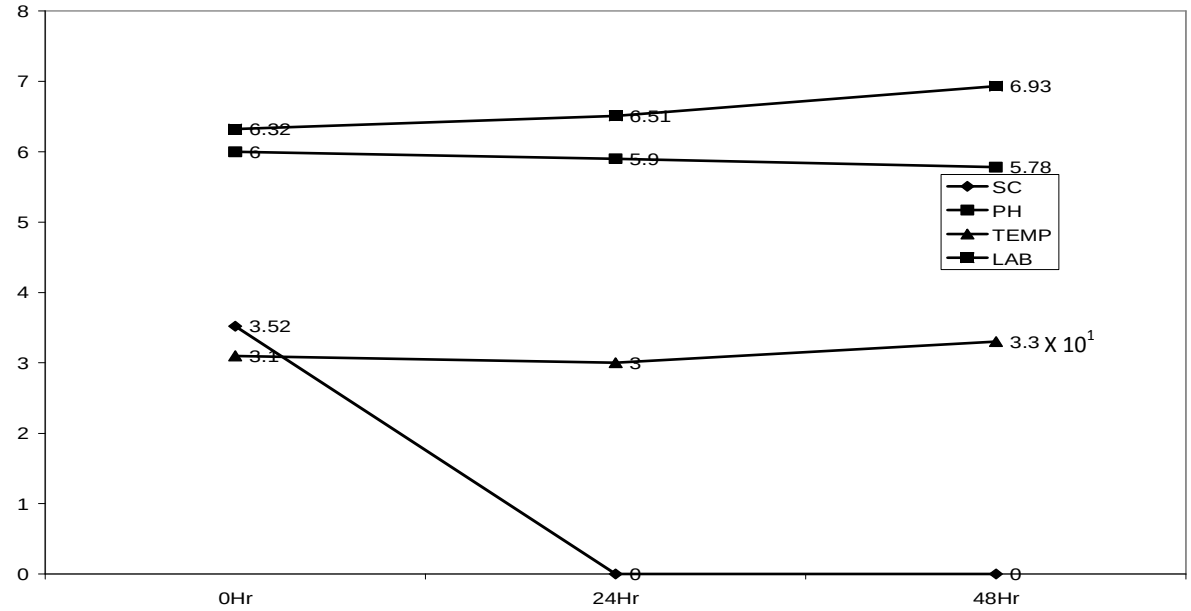

Figure 3: Mean staphylococcal counts of melon drink inoculated with *L. plantarum* (2.1 × 10^8 cfu/ml) stored for 48 hours at ambient Temperature.
Key: SC= Staphylococcal count, LAB= *L. plantarum* count

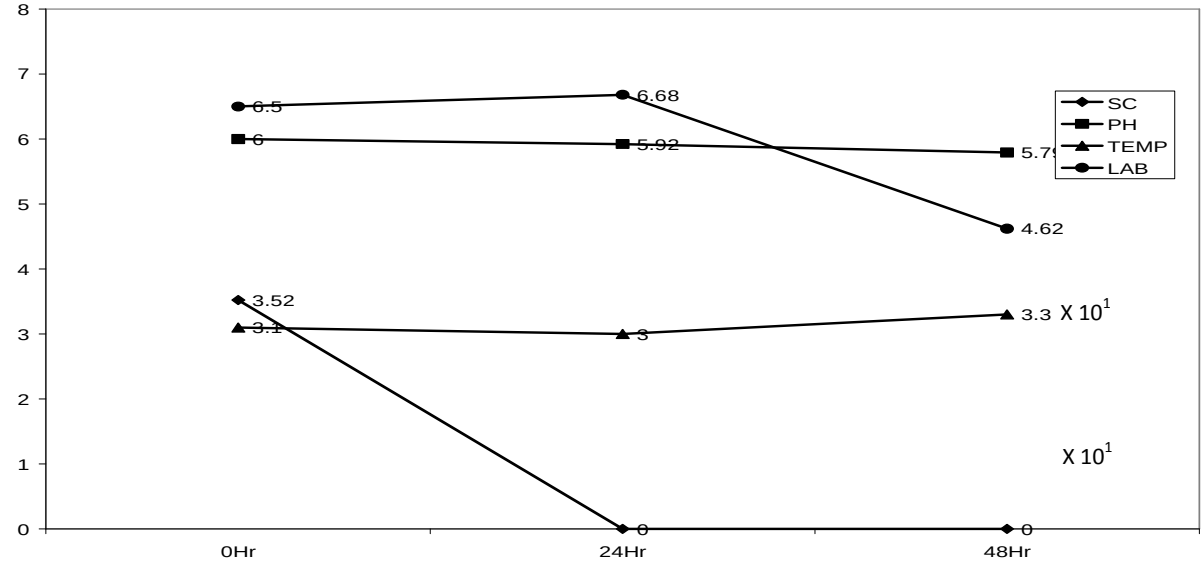

Figure 4: Mean staphylococcal counts of melon drink inoculated with *L. plantarum* (3.0 × 10^8 cfu/ml) stored for 48 hours at ambient Temperature.
Key: SC= Staphylococcal count, LAB= *L. plantarum* count

CONCLUSION

L. plantarum isolated from *Kunun zaki* exhibited antibacterial activities against *S. typhi* (24.66mm), *S. typhimurium* (34.33mm) and *S. aureus* (24.33mm). This study also showed that *L. plantarum* inoculated in melon drink has a sanitizing effect on *Staphylococcus aureus* by 3.52 Log count (cfu/ml) reduction.

RECOMMENDATIONS

It is recommended that further studies be carried out to isolate and characterize the antimicrobial compounds produced by the *L. plantarum* and to evaluate their probiotic potentials in the drink.

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