

Full Length Research.

# The antagonistic activities of Lactic acid bacteria isolated from Nigerian salad vegetables against methicillin resistant *Staphylococcus aureus*.

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Lactic acid bacteria (LAB) from different niches have been reported to exhibit antimicrobial activities against pathogens including the drug resistant ones. This study investigated the anti-methicillin resistant *Staphylococcus aureus* (MRSA) activities of LAB isolated from salad vegetables. 0.5 Mc Farland standard ( $10^8$  CFU/ml) of five confirmed MRSA strains, carrying *mecA* gene were challenged in the spot streak, agar well diffusion of the cell free supernatant (CFS) and soft agar (0.7%) overlay assays. Thirty-one (31) isolates of LAB from lettuce, cucumber and cabbage procured from eight market locations in Lagos, Nigeria were used. The isolated LAB were as follows; *Pediococcus pentosaceus*, *Weissella confusa* (cucumber), *Lactobacillus cellobiosus* (cabbage), and *Lactobacillus plantarum*, and *Lactobacillus salivarius* (lettuce). The degrees of inhibition against MRSA varied in different assays; in the spot streak, *P. pentosaceus* and *L. cellobiosus* from Festac and Isolo markets respectively exhibited widest zones of inhibition. In the agar overlay, *P. pentosaceus* and *W. confusa* from Mile 12 and Festac markets showed the widest zones of 28 mm and 24 mm respectively while the MRSA exhibited resistance to most of the CFS.

Although the particular inhibitory metabolites have not been elucidated, the LAB from salad vegetables have been found by this study to possess anti-MRSA abilities *in-vitro*.

**Key words:** Methicillin resistant *Staphylococcus aureus*, CFS, LAB, Antagonistic.

## Introduction

Lactic acid bacteria (LAB) are Gram positive, non-spore forming rods or coccoid forms that do not possess catalase, cytochrome oxidase and are strictly fermentative. They are characterized by an increased tolerance to a lower pH range and this enables them to outgrow other bacteria in the same natural fermentation system. Their antagonistic properties have been reportedly adduced to the production of metabolites like hydrogen peroxide, organic acids, bacteriocins, diacetyl, antifungal agents et cetera (Line *et al.*, 2008). LAB are responsible for inhibition of various pathogens (Tsai *et al.*, 2005; Adeniyi *et al.*, 2006; Schellenberg *et al.*, 2006; Line *et al.*, 2008; Vangelista *et al.*, 2010; Coda *et al.*, 2011). There are also different attempts to package them as "Probiotics" as follows; reduction of serum cholesterol

levels in mice (Ouweland *et al.*, 2002), improvement of lactose indigestion (Marteau *et al.*, 2001), adjunct to antibiotics (Hammad and Shimamoto 2010), immunomodulatory properties (Arienzo *et al.*, 2011).

Methicillin-resistant *Staphylococcus aureus* (MRSA) has been a clinical nightmare over the years since the introduction of methicillin to antimicrobial chemotherapy in 1960. MRSA is an important pathogen in nosocomial and even community acquired infections. It is usually resistant to more than one antibiotic including macrolides, clindamycin and fluoroquinolones, thus it has remarkable ability to acquire gene from other organisms through a lateral gene transfer (Kuroda *et al.*, 2001).

This antibiotic resistance underscores the fact that the era of antibiotics is fading out couple with the disruption

of normal flora balance and antibiotic-associated diarrhea (AAD) in up to 35% of treated patients (Beausoleil *et al.*, 2007).

The inhibitory activities of LAB against MRSA have been documented for instance, Barbara *et al.*, 2010, Fahad and Hala 2011 reported the inhibition of lipase and biofilm production while Schellenberg *et al.*, 2006 reported the in vitro anti-MRSA potentials of five strains of LAB (*Lactobacillus acidophilus* 8/4, *L. delbrueckii* ssp. *bulgaricus* 151, *L. salivarius* AWH, *Bifidobacterium animalis* B30, and *Streptococcus thermophilus* MK10).

Considering that salad vegetables are served raw and that there is little information on resident LAB on these niches, the aim of this study was to investigate the in vitro anti-MRSA activities of LAB isolated from Nigerian grown salad vegetables.

## Materials and Methods

The vegetables lettuce, cabbage and cucumber were purchased from eight (8) different market locations; Agboju, Festac, Idi- Araba, Isolo, Ketu, Mile 12, Oshodi and Yaba all in Lagos, Nigeria.

### Processing of vegetables

The vegetables were blended aseptically with NAKAI JAPAN magic and quilink blenders viz; the loading cup housing the blades of the blender was sterilized by autoclaving, allowed to cool to room temperature before loading each vegetable for blending.

### Isolation and identification of LAB

The blended vegetables (2g) were transferred into 18ml of sterile de man Rogosa Sharpe (MRS) broth in sterile falcon bottles, incubated for 12hr in candle extinction jar system at 37°C alongside sterile MRS broth serving as control. After 12 hr, inocula were transferred into MRS agar and incubated for up to 24 hr at the same conditions as above.

All the distinct colonies were subjected to catalase, oxidase (oxid, Australia) and Gram stain reactions. The Gram positive rods/ cocci, negative for oxidase and catalase were inoculated into API 50 CH (Biomérieux, France) sugar fermentation systems comprising forty-nine panel of sugars. The fermentation result was uploaded in apiweb vs 5.0 for interpretation.

### Sources, identification and Antibiogram of test pathogens

Four strains of MRSA were supplied by the Molecular Biology and Biotechnology Division of Nigerian Institute of Medical Research (NIMR).

These strains were previously identified by combination of cultural, biochemical and Polymerase chain reaction

(PCR) amplification of *mecA* gene. The antibiotic susceptibility test was done according to standard (CLSI, 2011).

Eight antibiotics of different concentrations in a multidisc were employed as follows; Chloramphenicol (CHL,10µg), Cloxacillin (CXC,5µg), Erythromycin (ERY,5µg), Gentamycin (GEN,10µg), Augmentin (AUG,30µg), Streptomycin (STR,10µg), Tetracycline (TET,10µg), Cotromoxazole (COT,35µg). Perfloracin (PER,30µg) in a single disc was included.

### Antagonistic assay LAB versus MRSA

This was done in three ways;

Soft agar overlay: LAB culture was overlaid with Mueller Hinton soft agar (0.7%) in which approximately  $10^8$  CFU/ml MRSA was added;

Spot streak: Pre- cultured, overnight, pure LAB isolate was spotted on the culture plate which has been previously seeded with 0.5 Mac Farland standard ( $10^8$  CFU/ml) of MRSA;

Agar well diffusion: 100µl cell free supernatant (CFS) of the LAB broth culture and control with read pH was introduced into well bored (using sterile cork borer, 6mm diameter) on Mueller Hinton agar which has been seeded with 0.5 Mc Farland standard ( $10^8$  CFU/ml) of MRSA.

The CFS was neutralized (approx. pH 7.0) by 1N NaOH and used for another set of antagonistic assay.

The assays above were incubated at 37°C for 24hr under aerobic condition and zones of inhibition measured in Millimeter.

## Result

Thirty- one (31) isolates of LAB identified as five (5) species, *Pediococcus pentosaceus*, *Weissella confusa*, *Lactobacillus cellobiosus*, *Lactobacillus plantarum* and *Lactobacillus salivarius* were isolated and these were common to particular vegetables (Table 1). The pH of CFS ranged from 4.16 to 4.83, while that of control did not really change. (Data not shown)

Based on the interpretive table, the four strains of MRSA showed multi resistance in vitro to Cloxacillin, Augmentin, Streptomycin, tetracycline and Cotrimoxazole. They were intermediately sensitive to erythromycin and perfloracin (Table 2).

The results of the soft agar overlay showed the LAB exhibiting varied spectra of inhibition against the test MRSA. *P. pentosaceus* isolated from Mile 12 market exhibited the widest inhibition range of 24-28 mm; this was followed by *L. salivarius* from Isolo with 22- 25 mm range (Fig. 2). The strains of MRSA were however resistant to *L. plantarum* (Table 3).

The spot streak assay showed inhibition by twenty- one (21) of the LAB isolates with *L. cellobiosus* and *W. confusa* from Yaba showing marked inhibition (Fig. 1). However, *L. cellobiosus*, *P. pentosaceus* from Idi- Araba and Mile 12 markets did not inhibit MRSA.

The four MRSA strains showed resistance to most of the acidic CFS and control except for the six (6) LAB isolates with marginal degrees of inhibition. The widest was 13 mm diameter and the narrowest 10 mm by *L. plantarum* and *P. pentosaceus* respectively. All the neutralized CFS did not inhibit the MRSA.

Table 1: The isolated LAB from the vegetables

Vegetable	LAB
Cucumber	<i>P. pentosaceus</i> <sup>5</sup> , <i>W. confusa</i> <sup>8</sup> ,
Cabbage	<i>L. cellobiosus</i> <sup>10</sup>
Lettuce	<i>L. plantarum</i> <sup>4</sup> , <i>L. salivarius</i> <sup>3</sup>

Note: The superscript represents the number of isolates

Table 2: Antibiogram of the test pathogens (MRSA)

Strain/ Antibiogram	Zones of inhibition (mm)								
	CHL	CXC	ERY	GEN	AUG	STR	TET	COT	PER
MRSA 1	20	R	18	15	R	15	14	R	18
MRSA 2	18	R	16	16	R	17	12	R	20
MRSA 3	18	10	19	15	R	17	11	R	20
MRSA 4	22	R	16	17	R	17	14	R	19

R- No zones of inhibition

Table 3: The soft agar overlay antagonistic assay of LAB versus MRSA

LAB vs MRSA	Inhibition zone diameter range (mm)			
	MRSA 1	MRSA 2	MRSA 3	MRSA 4
<i>P. pentosaceus</i>	24- 28	20- 25	18- 21	20- 22
<i>W. confusa</i>	21- 24	12- 18	12- 14	15- 19
<i>L. cellobiosus</i>	15- 17	18- 21	20- 22	10- 15
<i>L. plantarum</i>	0- 8	0-7	0	0
<i>L. salivarius</i>	20- 24	14- 18	22- 25	11- 14

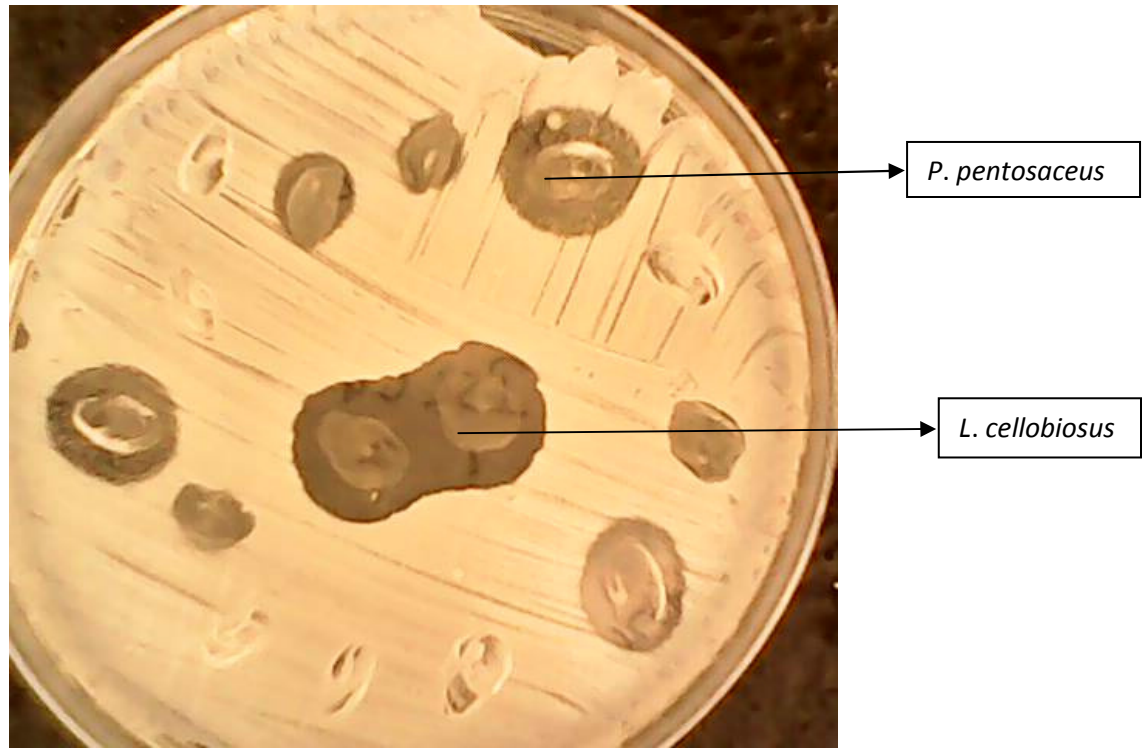


Fig. 1: Inhibition of MRSA by LAB on spot streak assay



Fig. 2: Soft agar overlay antagonistic assay of LAB against MRSA

## Discussion

MRSA is known to carry multidrug resistance genes as a result of its ability to acquire genes from other organism by lateral transfer (Kuroda *et al.*, 2001) hence the need to stem the problem of drug resistance. The gene mediating resistance to methicillin (*mecA*) was amplified previously in these strains of *Staphylococcus aureus* in our laboratory using PCR (Unpublished). The multidrug resistance of the study strains was underscored by their resistance to augmentin, streptomycin, tetracycline and intermediately sensitive to the quinolone, perfloracin while their possession of penicillin binding protein was shown by resistance to the  $\beta$ - lactam antibiotic (Cloxacilin). MRSA produces penicillin- binding protein which is encoded by *mecA* gene, carried on the Staphylococcal Cassette Chromosome *mec* (SSC *mec*) (Ito *et al.*, 2001).

The cell free supernatants of LAB isolated previously from these salad vegetables were inhibitory to some pathogenic bacteria in an agar well diffusion assay and the result indicated organic acid being responsible (Bamidele *et al.*, 2011). In the present study, the three assays employed were for comparison. However, the CFS of LAB spent culture marginally inhibited the MRSA and it was obvious that no other metabolites other than organic acids were responsible for the marginal inhibition seen against the MRSA as the neutralized CFS did not show any degrees of inhibition.

However, Fahad and Hala 2011 reported the anti- MRSA activities (up to 20 mm diameter) of acidic CFS from *L. acidophilus* isolated from vinegar. This acidic CFS also inhibited lipase and biofilm production by MRSA. In this study, the acidic CFS was from *L. plantarum* and *P. pentosaceus*.

It was also possible the metabolites that could otherwise be anti- MRSA were diluted in the CFS hence there is need to elucidate the array of metabolites produced in the spent cultures of these LAB. This will be considered in an ongoing study.

The results of both spot streak and soft agar overlay presented (Fig. 1 and Fig. 2) can stir up interest in the abilities by these LAB to respond to any threat in their environments. The cell- to- cell contact seemed to elicit the inhibitory potentials of these LAB as shown in this study. Although the particular inhibitory metabolites are being investigated in an ongoing study, it could be hypothesized that the metabolites are other than organic acids since not all the tested LAB exhibited this anti-MRSA activity.

The anti- MRSA activities of LAB have been reported by various workers. Nizin producing *Lactococcus lactis* subsp *lactis* was found to be antagonistic to one of four

MRSA (Park *et al.*, 2003). The effect was due to the bacteriocin.

On the other hand, Barbara *et al.*, 2010 reported that *L. acidophilus* and *L. casei* in cell- cell contacts to be more inhibitory to MRSA than their CFS. This was supported by the work of Schellenberg *et al.*, 2006. LAB seem to have the track record of being more antagonistic when in contact with pathogens. Savino *et al.*, 2011 demonstrated this in the assay of *L. delbrueckii* subsp. *Delbrueckii* and *L. plantarum* against gas producing coliforms.

## Conclusion

Salad vegetables, lettuce, cucumber and cabbage from our study sites are potent sources of lactic acid bacteria (LAB), and these LAB have anti- MRSA activities in vitro especially when they grow as co- culture with the pathogens. The particular inhibitory metabolites are other than organic acids.

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