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In vitro*, acidic, non-proteinaceous antifungal activities of lactic acid bacteria isolated from salad vegetables against human pathogenic *Candida albicans

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Abstract:

Background: The antagonistic abilities of lactic acid bacteria (LAB) against clinical isolates of *Candida albicans* are not quite widely reported and such are even scarce in Nigeria. This study therefore investigated inhibitory potentials of LAB isolated from locally grown cabbage, cucumber and lettuce against four (4) clinical isolates of *C. albicans*.

Methods: The cell free supernatants (CFS) generated from LAB culture filtrate was evaluated for anti-candida activity using agar well diffusion method, and the CFS-LAB pH was measured and neutralized using standard methods. The proteinaceous inhibitory metabolites were assayed for using sodium dodecylsulphate polyacrilamide gel electrophoresis (SDS-PAGE) technique. The LAB strains used were previously isolated and identified by 16S rRNA partial sequencing and their data submitted to GenBank for accessioning.

Results: The CFS of six (6) LAB strains showed varying degrees of anti-candida activity. *Pediococcus pentosaceus* BTA 51 from cucumber showed the widest inhibition zone of 14 mm while at neutral pH, it was 12 mm diameter. *Weissella confusa* BTA 20, BTA 40 isolated from cabbage and lettuce produced 10 mm and 12 mm zones of inhibition at acidic and neutral pH respectively. *Lactobacillus plantarum* BTA 07 from lettuce showed inhibition zone of 12 mm while *L. fermentum* BTA 47 and BTA 62 from cucumber showed zones of 14 mm each in acidic pH only. The SDS-PAGE did not detect any proteinaceous substances.

Conclusion: In conclusion, LAB isolated from cabbage, cucumber and lettuce produced organic acids, non proteinaceous metabolites at neutral pH, exhibiting *in vitro* inhibitory abilities against clinical isolates of *C. albicans*.

Keywords: *In vitro*, Lactic acid bacteria, 16S rRNA, antifungal, SDS-PAGE, salad vegetables

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In vitro*, l'acide, non-protéiques des activités antifongiques des bactéries lactiques isolées à partir de la salade de légumes contre les pathogènes de l'homme *Candida albicans

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Abstrait

Contexte: Les capacités antagonistes des bactéries lactiques (LAB) contre les isolats cliniques de *Candida albicans* ne sont pas très largement rapportées et sont même rares au Nigeria. Cette étude a donc examiné les potentiels inhibiteurs de LAB isolés de chou, de concombre et de laitue cultivés localement contre quatre (4) isolats cliniques de *C. albicans*.

Méthode: Les supernatants sans cellule (CFS) produits du filtrate de culture de LAB ont été évalués pour l'activité d'anticandida suivre la méthode de diffusion de puits d'agar et aussi bien que le CSF-LAB pH a été mesuré et neutralisé suivre des méthodes standard.. On a analysé les métabolites inhibiteurs protéineux pour l'usage de la technique de l'électrophorèse de gel de polyacrylamide de dodecyl sulfate de sodium (SDS-PAGE). Les tensions de LAB utilisées ont été précédemment isolées et identifiées par l'ordonnancement partiel du rRNA 16S et leurs données a soumis à GenBank pour accessioning.

Résultat: Le CFS de six (6) LAB tend des divers niveaux montrés d'activité d'anticandida. Le pentosaceus de pédiocoque BTA 51 de concombre a montré la zone d'inhibition la plus large de 14 millimètres tandis qu'à pH neutre, il était diamètre de 12 millimètres. Le confusaBTA 20, BTA 40 de Weissella a isolé dans le chou et la laitue a produit des zones de 10 millimètres et de 12 millimètres d'inhibition au pH acide et neutre respectivement, le lactobacille BTA plantarum 07 de laitue a montré la zone d'inhibition de 12 millimètres tandis que le fermentum de L. BTA 47 et BTA 62 de concombre montrait des zones de 14 millimètres chaque dans le pH acide seulement. Le SDS-PAGE n'a détecté aucune substance protéineuse

Conclusion: En conclusion, LAB isolé de chou, concombre et laitue a produit des acides organiques, des métabolites non protéiques à pH neutre, présentant des capacités inhibitrices invitro vis-à-vis des isolats cliniques de *C. albicans*

Introduction:

Lactic acid bacteria (LAB) found in different niches produce organic acids such as lactic, acetic, and other metabolites such as bacteriocins and hydrogen peroxide that have been shown to have antimicrobial activities (1, 2). Lactic acid produced in cell free supernatant by LAB and in co-culture has been shown to demonstrate anti-candidal activity. In a previous study, co-culture of LAB with *C. albicans* was reported to have led to loss of metabolic activity and eventually killing of the candida organism (2). In another study, four species of LAB belonging to *Lactobacillus* spp. and *Streptococcus thermophilus* showed varied inhibition against the fungal pathogen (3).

The protective effect of the LAB against infection with *C. albicans* in immunosuppressed Balb/c mice was demonstrated (4), while the anti-*Candida*

albicans effects of probiotic LAB, *L. rhamnosus* GR-1 and *L. reuteri* RC-14 was speculated to have been due mainly to lactic acid produced by LAB at low pH (5). Human LAB isolate, *L. fermentum* Ess-1 was shown to interfere with the growth of the pathogen in an agar-overlay and cell free *Lactobacillus* culture filtrate (LCF) (6). Coman et al. (7) also reported a study in which LAB, *L. rhamnosus* IMC 501 and *L. paracasei* in a liquid co-culture with *C. albicans* resulted in spectacular inhibition of the candida pathogen.

The abilities of vaginal LAB, *L. acidophilus*, *L. jensenii*, *L. crispatus*, *L. gasseri*, *L. johnsonii*, *L. vaginalis*, *L. agilis*, *L. ruminus* and *L. salivarius* to auto and co-aggregate with *C. albicans* were demonstrated by Gill et al. (8) with all the LAB able to auto and co-aggregate at varied degrees. *Lactobacillus crispatus* exhibited the highest degree of co-aggregation, while *L. jensenii* and *L. acidophilus* produced highest amounts of lactic acid and H₂O₂ respectively.

In some other studies, proteinaceous, acidic, and anti-inflammatory activities of LAB against *C. albicans* have been demonstrated. For instance, Shekh and Roy (9) characterized biochemically an anti-candida protein (ACP) produced by *E. faecalis* while some probiotic LAB were demonstrated to suppress expression of inflammatory gene associated with *C. albicans* infection (10). More recently, *L. plantarum* HS, *L. curvatus* HH, *P. acidilactici* HC and *P. pentosaceus* HM all isolated from honey were shown to exhibit antifungal activities against pathogenic *Candida* spp. in both agar well diffusion and soft agar overlay assays (11).

There is paucity of reports on LAB from salad vegetables against clinical isolates of *C. albicans*. This study was therefore designed to investigate the *invitro* activities and possible inhibitory metabolites of LAB isolated from cabbage, cucumber and lettuce against *C. albicans* isolated from clinical cases of vulvo-vaginal candidiasis (VVC).

Materials and Methods:

Sources of *C. albicans* and LAB

Four isolates of *C. albicans* from women with vulvo-vaginitis were supplied by the culture bank of Department of Medical Microbiology, College of Medicine, University of Lagos, Nigeria. The LAB isolates were obtained from the culture Bank of the Molecular Biology and Biotechnology Department of the Nigerian Institute of Medical Research (NIMR), Nigeria. These LAB isolates have been previously recovered from cabbage, cucumber and lettuce grown in Nigeria, identified by partial sequencing of their 16S rRNA gene and submitted to GenBank (NCBI) and European Nucleotide Archive (ENA) (Table 1).

Microbiology

The *C. albicans* were sub-cultured from the stock onto Sabouraud Dextrose Broth (SDB) and incubated at 37°C for 24 hours. Another inoculum was taken into Sabouraud Dextrose Agar (SDA) and

incubated at same atmospheres. Single colonies were Gram stained and every oval to round shaped cell was tested for the growth on germ tube as follows; 3 drops of fresh pooled human serum were put into a tube and with the aid of a sterile wooding applicator stick, a yeast colony was transferred into the serum and incubated for 3hrs at 37°C. A suspension of the culture was dropped on a clean microscope slide and cover slip placed on it. Using a high power objective, the presence of germ tube was confirmed.

The LAB isolates were sub-cultured in de Man Rogosa sharpe (MRS) agar to ascertain their purity. The typical LAB colonies with Gram positive reaction, and negative catalase, oxidase and spore reactions were used for anti-candida assay.

Centrifugation of MRS broth culture for bioassay test

All the LAB isolates were grown microaerophilically at 37°C in sterile MRS broth for 24 hours. The control which comprised of sterile MRS broth was incubated and treated the same way as the culture. The cultures and controls were subjected to cold centrifugation (4°C) at 10000g for 10min (Eppendorf, 5702 R). The supernatants were separated and filtered through a membrane (Millipore, 0.22µm) and the filtrates (cell free supernatant, CFS) used for anti-candida assay (12).

Acidity of cell free supernatants

This was measured by the use of pH meter (Thermo Electron Corporation, USA) after calibration at room temperature. The calibration was done using Thermo buffers of different pH and electrode storage solution.

Anti-candida assay

One hundred microliter (100µl) of the CFS was introduced into well bored (using sterile cork borer, 6mm diameter) on Mueller Hinton agar which has been seeded with 0.5 McFarland standard equivalent to 10⁸ colony forming unit per milliliter (CFU/ml) of *C. albicans* from

cultures on SDA. This was incubated in air at 37°C for 24 hours after which the zones of inhibition were measured in millimeter (mm).

The pH of MRS broth before inoculation with LAB, and the CFS of the broth culture after centrifugation/filtration were taken. The CFS was neutralized (pH 7.0) by 1N NaOH. The neutralized CFS was used for another round of antagonistic assay as stated above (13).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed on neutralized supernatants which showed inhibition, to rule out the presence of anti-candida protein (ACP). The CFS was treated with an equal volume of ice cold 20% trichloroacetic acid (TCA), incubated at 4°C for 30 minutes and then centrifuged for 20 minutes at 13,500 rpm. The resulting pellets were washed with acetone, re-suspended in 50µl SDS loading buffer, then boiled for 5 mins and centrifuged for 1 min at 10,000 rpm, before being placed on ice.

The electrophoresis was done according to system of Laemmli (14) using 12% (w/v) separating gel and 5 % (w/v) stacking gel in SDS-PAGE Model (BIORAD, UK). The low molecular weight standard (Fermenters SM 0661 protein ladder) was loaded alongside. The apparatus was connected with constant electric current (30mA) till the

bromophenol blue (BPB) reached the bottom of the plate (15), after which the gels were put into a container with staining solution containing 0.25% Coomassie brilliant blue R-250 dissolved in methanol with acetic acid and water (5:1:5). The destaining was done in a mixture of methanol, acetic acid and water (2:3:35, v/v/v) in a shaking water bath at room temperature until the bands became visible above the background.

Results:

The LAB produced metabolites inhibitory to test pathogen, *C. albicans* in *invitro* assays with *Pediococcus pentosaceus* BTA51 isolated from cucumber showing the widest zone of inhibition of 14mm diameter but with decreased (12mm) zone when neutralized CFS of the LAB was used. However, the CFS of 2 other LAB, *Weissella confusa* BTA20 from cabbage and *Weissella confusa* BTA40 from lettuce showed inhibition zones of 10mm and 12mm respectively in both acidic and neutralized CFS. Conversely, only acidic CFS of 3 other LAB showed anti-candida activities (Table 1). The result of SDS-PAGE showed no indication of any proteinaceous metabolites produced by the LAB and in the control.

Table1: Anti-candida activities of LAB strains from salad vegetables

LAB strain	Vegetable source	Accession number	Zone of inhibition (mm)	
			Acidic	Neutralized
<i>P. pentosaceus</i> BTA 51	Cucumber	MF580038	14	12
<i>W. confusa</i> BTA 20	Cabbage	MF580007	10	10
<i>W. confusa</i> BTA 40	Lettuce	MF580027	12	12
<i>L. plantarum</i> BTA 07	Lettuce	MF579994	12	-
<i>L. fermentum</i> BTA 47	Cucumber	MF580034	14	-
<i>L. fermentum</i> BTA 62	Cucumber	MF580049	14	-

- = No inhibition, mm = millimeter

Discussion:

The anti-candida investigations done in this study employed acidic and neutralized CFS of the LAB. This was to determine by elimination method, the inhibitory metabolites whether organic acids as reported by Adeniyi and Iveren (16) in Nigeria or others. While *L. fermentum* in the study of Adeniyi and Iveren (16) did not inhibit *C. albicans*, the *L. fermentum* BTA47 and *L. fermentum* BTA62 used in this study both exhibited the widest inhibition and this was demonstrated to be due to the effect of low pH. The pH of their CFS was 4.7 (unpublished). This was in tandem with the work of Manzoor et al (17). The yeast tested in the study of Adeniyi and Iveren (16) was standard strain instead of clinical isolates used in the present study. It is also worth noting that LAB isolates in our study have clearly identified by 16S rRNA partial sequencing and data submitted to GenBank.

Elsewhere, the CFS of human isolate of *L. fermentum* Ess-1 exhibiting some probiotic potentials was found to inhibit vulvo-vaginal *Candida albicans* (VVC), although non-acidic inhibition was demonstrated, proteinaceous metabolites were not excluded (18). To the best of our knowledge, this is the first study in Nigeria to demonstrate non-acidic, non-proteinaceous inhibition of clinical isolates of *C. albicans* by LAB from salad vegetables. The activities of *P. pentosaceus* BTA51, *W. confusa* BTA20 and *W. confusa* BTA40 were not due to proteinaceous metabolites as SDS-PAGE did not indicate presence of any protein. While the last 2 LAB species produced solely non-acidic anti-candida metabolites, the acidic CFS of *Pediococcus pentosaceus* BTA51 seemed to have a marginal anti-candida effect.

The SDS-PAGE was used in this study such that the size and number of any proteinaceous substances produced can be determined otherwise a protease such as trypsin or pepsin would have sufficed to rule out the effect of proteinaceous substances against the

fungus. In studies by Shekh and Roy (9), Graham *et al* (19) and Ishijima *et al* (20), anti-candida activities of proteinaceous substances such as bacteriocins were reported while Lade *et al* (21) on the other hand, demonstrated lack of bacteriocin activities produced by *L. lactis* and *L. plantarum* against *C. albicans*.

Although, the mechanisms of inhibition of *C. albicans* by LAB have been reported to be poorly understood, various authors have demonstrated biofilm inhibition, anti aggregation, co aggregation, nanoparticle enhancement, suppression of *C. albicans* induced factors, anti-adhesion, anti-candida adjunct and many others (8-10, 22-25).

In conclusion, the acidic and non proteinaceous metabolites produced by LAB isolated from cabbage, cucumber and lettuce grown in Nigeria exhibited *invitro* inhibitory abilities against clinical strains of *C. albicans* while *W. confusa* BTA20 and *W. confusa* BTA40 in particular produced non-proteinaceous anti-candida factor at neutral pH.

Authors' contributions:

BAA and TAB conceptualized and designed the study; TAB performed field and laboratory work; TAB and SIS prepared the manuscript draft; TAB, BAA and SIS reviewed the final draft. All authors approved the final manuscript.

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