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Research Article

Antifungal Capacity of Lactic Acid Bacteria Isolated From Salad Vegetables

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ABSTRACT: This study explores the use of lactic acid bacteria from fresh salad vegetables to inhibit fungal growth. The antifungal assay was done using the agar well diffusion method as reported by Schillinger and Lucke (1989). The largest zone of inhibition (25mm) was recorded by the antagonistic activity of the isolate identified to *Lactobacillus plantarum* against *Candida albicans* ATCC 90029. No activity was recorded against *Candida parapsilosis* ATCC 22019, *C. valida* UCH 1508, *C. pseudotropicalis* UCH1408, *C. tropicalis* UCH 1308 and *Trichophyton interdigitalis* UCH1708. The cell free supernatant (CFS) of the isolate described to be *Lactobacillus brevis* was exceptional as it was the only CFS that inhibited the growth of *Epidermophyton floccosum* UCH 1908. The results show that LAB isolated from salad vegetables can inhibit some fungi pathogens by developing zones around agar well that contain LAB metabolites and can probably be a feasible option for the chemotherapy of fungal infections given the drug resistance exhibited to antifungal agents currently in use.

Keywords: Antagonistic activity, lactic acid bacteria, salad vegetables, *Candida*, dermatophytes.

INTRODUCTION

Lactic acid bacteria (LAB) are a group of physiologically related fastidious and ubiquitous Gram positive organisms. They are also one of the most prominent non – pathogenic bacteria that play a vital role in our everyday life from fermentation, preservation and production of wholesome foods, and vitamins to prevention of certain diseases and cancer due to their antimicrobial action (Afolabi *et. al* 2008). Fresh vegetables constitute a good source of lactic acid bacteria with the ability to inhibit wide range of spoilage fungi (Sathe *et al.*, 2007) and this property is currently being employed as a biopreservative that will help in prolonging the shelf – life of fresh vegetables. This is possible due to the production of a variety of antagonistic factors that include metabolic end products – ethanol, formic acid, acetone, hydrogen peroxide, diacetyl – and bactericidal proteins termed bacterocins which are produced as a natural competitive means to overcome other microorganisms sharing other niche. On the other hand, fungal diseases are commonplace in man and the contribution of drug resistance among microorganism results in serious health implications. Therefore this paper reports investigations into the antagonistic activities of some members of the lactic acid bacteria group against clinical isolates of some *Candida* sp. and dermatophytic species.

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MATERIALS AND METHODS

Sample collection and preparation

Fresh samples of five salad vegetables namely: carrots, lettuce cucumbers, parsley and cabbage were purchased from local markets at Bodija, Ojoo, Dugbe, Sango and vendor salad stands at Bodija and Mokola all in Ibadan, South – Western Nigeria. The samples were collected

in plastic bags and transported to the laboratory for microbiological analysis the same day. In the laboratory samples were prepared by aseptically chopping, into small bits, each of the vegetables to minimize contamination before adding them to sterile peptone water and leaving it overnight.

Isolation, Identification and Characterization of LAB Isolates

Each sample bottle containing the various vegetables in peptone water was mixed thoroughly and 1ml was taken using a sterile pipette to prepare a serial dilution with sterile distilled water. The pour plate method was used for the isolation by transferring aseptically 0.1ml of each dilution into sterile plates containing deMan Rogosa Sharpe (MRS) agar and incubated anaerobically for 24hours at 37^0C. After growing, a single colony was tested and examined morphologically and microscopically for purity and then subcultured in MRS broth. General LAB identification and characterization was carried out by employing macroscopic, microscopic, physiological and biochemical tests.

Test Organism

The test organisms used in this study were both ATCC strains and clinical strains obtained from Medical Microbiology unit, University College Hospital, Ibadan, Nigeria. They were maintained on Tryptone Soy Agar slants in a refrigerator and sub cultured after two weeks.

Antibiotic susceptibility test

Antibiotic susceptibility test for each fungal pathogen was performed using the agar well diffusion method. A 0.1ml actively growing broth culture containing 1x 10^6 cfu/ml of each fungal pathogen was spread using a spreader on already set Muller Hinton agar. Using a cork borer of diameter 8mm, wells were bore on the agar and different antibiotics were introduced into the wells. Each well contained the following antifungal agents: clotrimazole (2,000µg/ml), nyastatin (100,000IU units/ml), ketoconazole (2,000µg/ml) and griseofulvin (1000µg/ml). The plates were incubated at 25^0C for 24 - 48h. After this period, the diameter of the zone of inhibition of each disc was measured. The zone of inhibition corresponded to the antibiotic activity of each antifungal (Norrby, 1992). Resistance was defined by the absence of a zone of inhibition. The relative susceptibility of each test fungi to each antibiotic was shown by a clear zone of inhibition. Drug for use in sensitivity testing were obtained from a pharmacy store in Ibadan.

Determination of the *in vitro* activity of LAB isolates

Preparation of cell free supernatants

Cell free supernatants (CFS) of the lactic acid bacteria isolates were obtained by centrifuging, at 10,000 x g for 10mins, MRS broth cultures of 24, 48 and 72 h anaerobic incubation at 37^0C (Ogunbanwo, 2005)

***In vitro* Inhibition Assay**

The antagonistic activity of cell free supernatants of the selected LAB against the pathogens used was done using the agar well diffusion technique. The plates were incubated at 25^0C for 24 - 48h after which they were examined for clear zones around the wells. The experiment was carried out in duplicates. The antimicrobial effect was recorded by measuring the zone of inhibition around the well.

RESULTS

A total of 35 strains of lactic acid bacteria were randomly picked from various vegetables. The isolates were subjected to various morphological, physiological and biochemical tests. The isolates were identified as *Lactobacillus plantarum, Lactobacillus cellebiosuis, Lactobacillus delbruesckii, Lactobacillus fermentum, Leuconostoc mesenteriodes, Lactobacillus brevis, Lactobacillus casei* and *Pediococci* spp. The percentage occurrence of lactic acid bacteria obtained from the salad vegetables used in this study reported *Leuconostoc mesenteroides* as having the highest occurrence with 26. 47%. It was followed by *Pediococci* spp which had 11.43%, *Lactobacillus plantarum*, 14.70% and *Lactobacillus cellebiosuis*, 2.94% (Table 1).

Table 1:
Number and percentage of occurrence of LAB species isolated from salad vegetables

Isolates	Number	Percentage
L. mesenteroides	9	26.47
L. brevis	4	11.76
L. delbrueckii	4	11.76
L. fermentum	2	5.88
L. plantarum	5	14.70
Pediococci Spp	7	20.59
L. cellebiosuis	1	2.94
L. casei	1	2.94
Total	34	100

Table 2:
Antibiogram showing susceptibility or resistance to various antifungal agents

Organisms	**Antifungal agents**			
	Griseofulvin (1000µg/ml)	**Nystatin** (100,000IU/ml)	**Ketoconazole** (2000µg/m)	**Cotrimozaole** (2000µg/ml)
Candida valida UCH 1508	R	R	R	R
C. pseudotropicalis UCH 1408	R	R	R	R
C. tropicalis UCH 1308	R	R	R	R
C. glabrata UCH 1208	R	R	R	R
Trichophyton rubrum UCH 1608	R	R	R	R
Trichophyton interdigitalis UCH 1708	R	R	R	R
Trichophyton tonsurans UCH 1808	R	R	R	R
Epidermophyton flocussum UCH 1908	R	R	R	R
C. albicans ATCC 90029	R	R	R	R
C. parapsilosis ATCC 22019	R	R	R	R
C. krusei ATCC 6825	R	R	R	R

Table 3:
Antagonistic effect of LAB metabolites (MM) against standard and clinical fungi by agar well diffusion

LAB ISOLATES	*Candida valida UCH 1508*	*C. pseudotropicalis UCH 1408*	*C. tropicalis UCH 1308*	*C. glabrata UCH 1208*	*Trichophtyon ruburm UCH 1608*	*T. interdigitalis UCH 1708*	*T. tonsurans UCH 1808*	*Epidemophton floccosum UCH 1908*	*C. albicans ATCC 90029*	*C. krusei ATCC 6825*	*C. parasilopsis* ATCC 22019
L. mesenteroides	-	-	15±0.0	20±1.0	13±0.5	-	14±0.0	-	15±1.0	-	-
L. brevis	-	-	15±1.0	-	15±1.0	-	-	14±1.0	-	-	-
L. fermentum	-	-	-	-	15±0.0	-	-	-	-	-	-
L. delbruesckii	-	-	-	13±0.5	11±2.0	-	-	-	24±0.0	-	-
L. plantarum	-	-	-	14±0.0	11±2.0	-	15±1.0	-	25±0.0	17±0.0	-
L. cellebiosuis	-	-	-	-	14±1.0	-	11±2.0	-	23±0.0	-	-
L. casei	-	-	-	-	-	-	-	-	-	-	-
Pediococci spp	-	--	-	16±0.1	16±0.0	-	11±2.0	-	20±0.0	20±0.0	-

Diameter of cork borer = 8mm
- = absence of antagonistic activity

All fungal test organism used (both ATCC strains and clinical strains) demonstrated 100% resistance to all the antifungal used (Table 2) even at very high concentrations.

The cell free supernatant (CFS) of LAB isolates produced antagonistic activities against all the pathogens used in this study except for *Candida parapsilosis* ATCC 22019, *C. valida* UCH 1508, *C. pseudotropicalis* UCH 1408, *C. albicans* UCH 1008 and *Trichophyton interdigitalis* UCH 1708. The largest zone of inhibition (25mm) was produced by *Lactobacillus plantarum* against *C. albicans* ATCC 90029 (Table 3). The CFS of the isolate identified to be *L. brevis* was exceptional as it was the only isolate that was able to exhibit antagonistic activity against *Epidermophyton floccosum* UCH 1908. *Trichophyton rubum* UCH 1608 and *T. tonsurans* UCH1808 were the most susceptible dermatophytes to metabolites produced by the LAB isolates. *The largest* zone of inhibition against the dermatophytes was shown by the isolate identified to be *Pediococci* against *Trichophyton rubum* UCH 1608. The isolate identified as *L. fermentum* did not inhibit *Candida glabrata* UCH 1208.

DISCUSSION

Varngm (2002) noted that versatility is an important attribute of lactic acid bacteria and although nutritionally fastidious, they are able to colonize a wide range of environments. This explains their presence in the tested salad vegetables. Mundt *et al*., (1967) also reported the presence of a variety of lactic acid bacteria including *Leuconostoc* species in crop plants. *Leuconostoc mesenteroides* was identified as the predominant LAB specie. This is consistent with Pederson and Albury (1969). The varying percentages of occurrence of LAB isolates indicate a wide distribution of LAB in fresh vegetables of different origin. Studies have shown that treatment with antifungal drugs often results in the appearance of resistant strains of fungi (Hampton, 2008). Fungi multi drug resistance is caused by the increased expression of genes that encode nonspecific drug-efflux pumps belonging to the ABC family of transporter proteins such as Pdr5p (Balzi *et al.*, 1987).

Early investigations have shown the antagonistic activities of LAB against fungal pathogens. Ronnqvist *et. al*, (2007) reported that a LAB isolate, *L. fermentum* Ess – 1 showed activity against *C. glabrata* and *C. albicans*. No activity was demonstrated against *C. pseudotropicalis*. This confirms the works of Gulahmadov *et al.*, (2009). Trias *et al.*, (2008) reported that LAB isolated from fresh fruits and vegetables can act as biocontrol against phytopathogenic bacteria and fungi and *L. mesenteroides* had the best antagonistic capacity when tested against all the phytopathogenic fungi. Studies on antifungal LAB reveal that the production of fungal inhibitory substances occurs among many different species. Species belonging to the genus Lactobacillus are reported in the majority of studies (Sathe *et al.*, 2007). It is not known if this is due to the fact that they generally possess more antifungal capacity than other species or whether it is due to higher prevalence in the type of environment studied. A comparison of the different inhibition zones produced by the LAB isolates belonging to each LAB genus showed that *Lactobacillus* species were the most inhibitory to the test organisms followed by *Pediococci* and *Leuconostoc* spp in that order.

The inhibitory activity of the LAB isolates has been attributed to the production of lactic and acetic acid, hydrogen peroxide, and diacetyl (Ogunbanwo, 2005; Adeniyi et al., 2006; Adeosokan *et al.*, 2008; Sathe *et al.*, 2007; Afolabi *et al.*, 2008; Collins & Aramaki, 1980). The general mechanism behind the inhibition is suggested to be due to the passage of undissociated acid molecules across the cell membrane. Once inside the cell, the higher pH of the cytoplasm will lead to dissociation of the acid. This will generate an accumulation of the anion of the acid together with protons and consequently a decrease of the intracellular pH (pH_{int}). In yeast a lowering of pH_{int} has been shown to inhibit glycolysis. Thus, intracellular acidification directly affects growth. Apart from the lowering of pH_{int}, other actions, such as anion accumulation and disordering of cell membrane structure have also been proposed as being responsible for the weak acid inhibition. Most studies of the effects of weak acids on fungal growth have established that a certain pH is necessary for the inhibitory action, whereby the acid is undissociated, leading to diffusion across the membrane (Sathe *et al*., 2007). Condon (1987) reports that hydrogen peroxide could in some cases be a precursor for the production of other potent antimicrobial species such as super oxide and hydroxyl radicals. Diacetyl is important for the organlopetic quality of food products. Diacetyl is known to be effective against yeasts and molds. At a pH greater than 7.0, it is found to be ineffective and effective at pH less than or equal to 7.0 (Jay, 1996). Apart from the actual inhibition of fungal growth, LAB can also specifically inhibit production of mycotoxins (Gourama & Bullerman, 1997) or immobilize mycotoxins through binding to their surface.

Since the clinical use of antibacterial drugs, immunosuppressive agents after organ transplantation, cancer chemotherapy, and advances in surgery are associated with increasing risk of fungal infections and following this increased frequency of systemic fungal diseases is the increased in antifungal drug resistance, novel approaches are needed in the development of new chemotherapeutic agents for fungal infections. It has been demonstrated from this study that LAB isolated from fresh salad vegetables have a high potential for the treatment of *Candida* and dermatophytic infections.

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