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Antifungal Activity of Bacteriocins of Lactic Acid Bacteria from Some Nigerian Fermented Foods

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Abstract: This study was conducted to evaluate the antifungal activity of bacteriocins of lactic acid bacteria from some Nigerian fermented foods (Eko, Fufu, Iru and Ogi). Screening of 25 bacteriocin-producing Lactic Acid Bacteria (LAB) isolated from Eko, Fufu, Iru and Ogi against three spoilage fungi (*Penicillium citrinum*, *Aspergillus niger* and *A. flavus)* associated with stored fermented foods revealed that 68% of the isolates possessed antifungal activity. About 36% of those which showed a high fungicidal activity were isolated from sorghum Ogi. *Penicillium citrinum* was the most sensitive while *Lactobacillus brevis* SG1 was the most active of all the LAB strains. Spore germination and mycelia growth of *P. citrinum* was inhibited in the presence of both the LAB cells and their neutralized, cell-free culture supernatants (CFNS) using different bioassays. Generally, the results of all the bioassays were comparable. However, the antifungal activity of the LAB strains was easily detectable in liquid media and more pronounced on spore germination of the fungus than on its mycelia growth. The inhibitory effect of the bacteriocins on spore germination of *P. citrinum* ranged from 2.44 to 85.36% in *Lactobacillus casei* FF1 and *L. brevis* SG1, respectively. This study demonstrated that germination of fungal spores and fungal growth were significantly reduced by the LAB cells and/or their cell-free filtrates, thus indicating the propensity of the use of these antifungal substances in bio-control.

Key words: Bio-control, fungal inhibition, *Penicillium citrinum*, bioassay, growth inhibition

INTRODUCTION

Fermented foods are largely consumed in Africa where they constitute a bulk of the diet (Adebayo and Aderiye, 2007). They have also been reported to constitute 25% of the diet of Europeans and 60% in many developing countries (Stiles, 1996). Examples include Thao Nao from Northern Thailand, Kinema from India and nato from Japan (Chukeatirote *et al*., 2006). Majority of Nigerian fermented foods are products obtained through lactic acid fermentation. This is desirable because apart from improving the organoleptic properties of foods, fermentation also reduce spoilage of food due to the production of antimicrobial compounds such as organic acids, diacetyl, hydrogen peroxide and bacteriocin during fermentation (Ogunbanwo *et al*., 2004).

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The production of many indigenous African foods is often plagued by premature spoilage due to their high moisture content. Earlier studies had shown that spoilage of fermented foods in Nigeria is mostly due to fungal activity especially moulds which proliferate at low pH (Adebayo, 2007). Activity of spoilage fungi had been reported to cause a reduction in mineral nutrients and quality of Nigerian fermented foods (Teniola and Odunfa, 2002; Aderiye *et al*., 2006).

The problem of fungal spoilage of food is of great concern in the food industry and many researches have been done to minimize this (Soomro *et al*., 2002). There is an urgent need to prevent fungal contamination of these foods, because it could cause economic and/or health hazards due to loss in nutritional and organoleptic properties and/or production of mycotoxins (Pitt and Hocking, 1999). Fungal spoilage of food is a world-wide phenomenon. In Western Europe, mould spoilage of bread alone was estimated to cause an annual loss of about 242 million pounds (Corsetti *et al*., 1998). Also fungal spoilage of date-palm fruits of Saudi Arabia has been reported by Al-Sheikh (2009).

Many food preservation techniques including physical, chemical and biological methods have been explored as antidote to food spoilage. While the use of physical methods is plagued with the problem of loss of nutritive values in foods due to the effect of heat, that of chemical methods has the disadvantage of producing side effects like cancer or toxins on the consumer (Davidson, 2001). Therefore, recent advances in the use of biological methods involving naturally occurring antimicrobials in foods to prevent microbial spoilage have increased as their use has been shown to retain the nutritive value of food and present no side effects on the consumer (Klaenhammer, 1993).

Lactic Acid Bacteria (LAB) are Gram-positive, catalase-negative, non-motile, non-spore forming, aciduric bacteria. They are found in carbohydrate-rich materials, especially fermented foods (Soomro *et al*., 2002). They are of great interest in recent times because of their ability to produce antimicrobial substances like bacteriocin, hydrogen-peroxide and organic acids (Stiles, 1996). Bacteriocin is the most potent of all the antimicrobial compounds produced by Lactic Acid Bacteria (LAB). Bacteriocins are ribosomal synthesized peptides which are generally only active against closely related bacterial species (Klaenhammer, 1993). Bacteriocins produced by LAB are of global interest to the food fermentation industry because they inhibit the growth of many spoilage and pathogenic bacteria and thus extend the shelf life of foods (Herrero *et al*., 1996; Vescovo *et al*., 1996; Ogunbanwo *et al*., 2004; Mathieu *et al*., 2008). Whereas a lot of literature is available on the antibacterial activity of bacteriocins from LAB (Seuk-Hyun and Cheol, 2000; Mataragas *et al*., 2002; Adebayo and Aderiye, 2007), there are only few reports on their antifungal activity. Lavermicocca *et al*. (2000) attributed the antifungal activity of *Lactobacillus plantarum* isolated from sour dough to the action of organic acids like phenyl lactic acid and 4-hydroxyl-phenyl-lactic acid. Vanne *et al*. (2000) showed that the growth of toxigenic storage fungi was restricted by LAB *in vitro* and attributed this to the combined effect of lactic acid and bacteriocin. Meanwhile, Magnusson and Schnurer (2001) found that *L. coryneformis* possessed an antifungal activity attributed to the production of reuterin, a bacteriocin with broad spectrum antimicrobial activity.

There is need to examine the use of a biological method to control fungal spoilage of fermented foods. The use of bacteriocinogenic LAB cells as starter cultures or the incorporation of their bacteriocins into fermented foods could be a way out. This study therefore reports the influence of bacteriocin of LAB isolates from some Nigerian fermented foods on spoilage fungi. This probably is the first report on the antifungal activity of bacteriocins from Nigerian fermented foods.

MATERIALS AND METHODS

The study was carried out at the Microbiology Unit of the Department of Science Technology, The Federal Polytechnic, Ado-Ekiti and the University of Ado-Ekiti, Nigeria during February 2009 to May 2009.

Source of Organisms

The isolation and identification of the Lactic Acid Bacteria (LAB) cultures were as previously described (Adebayo and Aderiye, 2008). A total of 96 lactic acid bacteria strains were isolated from Eko, Fufu, Iru and Ogi, indigenous Nigerian fermented foods, produced from maize, cassava, locust beans and sorghum ogi respectively. These were identified and characterized according to the scheme of Holt *et al.* (1994) using morphological and biochemical tests. They were later screened for antibacterial activity against a wide array of indicator organisms (20) using the agar spot and agar well method. Cultures of 25 of this LAB which showed broad spectrum towards the test bacteria were used for this study. The LAB cultures were maintained as frozen stocks at -22°C in MRS broth until when needed. The antimicrobial substances (AMS) produced by the 25 LAB strains was purified characterized and screened for antifungal activity.

Fungal species commonly associated with spoilage of stored fermented foods were isolated as described by Ghildiyal and Pandey (2008) and used as test organisms. The fungal and LAB strains were cultured and maintained on potato dextrose and de Mann, Rose and Sharpe (MRS) media (Oxoid, London), respectively.

Production and Purification of the Antimicrobial Substance

The antimicrobial substances were produced as previously described (Adebayo and Aderiye, 2008). They were purified by ammonium precipitation and ion-exchange chromatography as described by Bonade *et al.* (2001).

Characterization of the Antimicrobial Substance (AMS)

Sensitivity to proteolytic enzymes was tested by treating both crude supernatant fluid and purified AMS with pepsin, proteinase K, pronase E and trypsin. Residual activity was determined by the agar diffusion method as described by Matagaras *et al.* (2002). Heat sensitivity was assessed by testing the residual activity of the supernatant fluid after treatment at 100°C at 121°C for 5 to 60 min. Further characterization of the AMS is reported by Adebayo and Aderiye (2008).

Preparation of Culture Filtrates

The bacteriocin-producing LAB strains were grown anaerobically (Gaspak, BBL) at 30°C for 24 h. Supernatant fluid was collected by centrifugation, adjusted to pH 7, treated with 5 mg mL^{-1} catalase to eliminate the effect of lactic acid and hydrogen peroxide, respectively (Bonade *et al.*, 2001) and filter sterilized (0.2 μm pore size; Millipore). The sterile Cell-Free Neutralized Supernatant (CFNS) was either kept at 4°C or freeze-dried. Freeze-dried samples were usually re-suspended (to a 10-fold concentration) in 20 mM citrate buffer (pH 7.0) whenever needed.

Preliminary Screening for Antifungal Activity

The LAB strains were screened for antifungal activity against three spoilage fungal strains of stored fermented foods; *Aspergillus niger* FF2 from Fufu, *Penicillium citrinum* EK1 from Eko and *Aspergillus flavus* EB3 from Eba using the agar-well diffusion method. The

fungal spore suspension was obtained and concentration determined as previously described (Aderiye *et al*., 1989). Sterilized Potato Dextrose Agar (PDA) was prepared and allowed to solidify in Petri dishes. The surface of each plate was inoculated with 1 mL of spore suspensions containing approximately 4×10^5 spores mL^{-1} each of *Aspergillus niger* FF2, *Penicillium citrinum* EK1 and *Aspergillius flavus* EB3 and left to stand for 30 sec. After this, wells (5 mm diameter) were cut into the agar plate using a sterile cork borer, sealed with a drop of sterile agar to avoid leakage and 100 μL of neutralized cell-free culture supernatant fluid of each LAB strain was placed into each well. Plates were pre-incubated for 2 h at 4°C to allow a diffusion of the CFNS and then incubated at 25°C for 24 to 48 h. The antifungal activity was determined by measuring the diameter of inhibition zone around the wells.

Those that showed inhibitory zones against any of the fungi were recorded. A reversion of the zone of inhibition after 96 h was recorded as a fungistatic action while those with inhibition zone for at least 7 days were recorded as being fungicidal in action. Four replicates were used and the experiments were performed thrice to confirm the results.

Inhibition of Fungal Spore Germination

The neutralized cell-free culture filtrate of each LAB isolate was screened against the spores of *P. citrinum* EK1 using the method of Walker *et al.* (1996). In this assay, approximately 250 μL of CFNS of each LAB cell and 500 μL of sterile Potato Dextrose Broth (PDB) containing 4×10^5 *P. citrinum* spores per milliliter was added to sterile Eppendorf tubes. The control experiment involved the addition of sterile MRS broth only to the fungal spore suspension. The contents of the vials were mixed for 10 sec and incubated at 25°C for 24 h. After incubation, the vials were vortexed and aliquots of the spore preparation were introduced into a haemocytometer (Weber) and observed under a glass cover slip with the microscope (x400 magnification). The total number of spores and those that had germinated were counted and the percent germination of the fungal spores was determined. A spore was deemed to have germinated if the germ tube had emerged.

All the experiments were carried out in triplicates and conducted twice.

Inhibition of Fungal Growth

Ten milliliter of sterile PDB containing 10^5 spores of *P. citrinum* mL^{-1} and 2 mL each of CFNS of the LAB isolates were added into triplicate 100 mL Erlenmeyer flasks. In the control experiment, only 2 mL of sterile MRS broth was added to the PDB containing 10^5 spores of *P. citrinum*. The flasks were incubated at 25°C with agitation (100 rev min^{-1}) for 5 days. The absorbance was measured every 24 h at 490 nm to assess the growth of *P. citrinum* in the presence of the CFNS.

The percentage inhibition was determined by comparing the growth of the control with the treated fungi. The dry weight of the mycelia was determined after incubation for 5 days by drying the sediment on a pre-weighed filter paper in an oven at 70°C for 48 h. All determinations were done in triplicates and the means were calculated. The percent inhibition was calculated using the formula:

$$R_1-R_2/R_1$$

where, R_1 was the dry weigh of control and R_2 was the dry weight of the treated fungus.

Statistical Analysis

The data obtained were analyzed by ANOVA and tests of significance were determined by Duncan's Multiple Range Test (DMRT) (Steel and Torrie, 1981).

RESULTS

The sources of 25 strains of bacteriocin-producing LAB are shown in Table 1. The LAB strains were majorly (76%) rods. The predominant species was *Lactobacillus plantarum.* Forty percent of the isolates were from sorghum Ogi while cooked Fufu recorded the least (4%).

Characteristics of the antimicrobial substances produced by the LAB strains showed that they are protein and strongly heat stable (Adebayo and Aderiye, 2008) (data not shown). Due to their high insensitivity to heat as evidenced in Fig. 1 and in accordance with the description of Savadogo *et al.* (2006) they should be regarded as bacteriocin.

A high percentage (68%) of Lactic Acid Bacteria (LAB) was observed to possess antifungal activity, inhibiting at least one of the three spoilage fungi within two days of inoculation. Generally, there was a daily increase in the diameter of the inhibition zones up to 96 h after which there was a slight reduction (Table 2). None of the LAB isolates recorded 100% level of inhibition on the spores of *P. citrinum.* However, bacteriocins of all the LAB isolates with the exception of *Lactobacillus plantarum* EK1, *L. plantarum* EK4 and *L. fermentum* SG10 exhibited varied reducing effects on the germination of spores of *P. citrinum* suggesting the variability of bacteriocins from the LAB cells as shown in Table 3. The result of the turbidity measurement (OD_{490}) of the growth of *P. citrinum* in the presence of CFNS of LAB isolates is shown in Table 4. Turbidity measurement of the various LAB isolates differed significantly ($p<0.05$). Generally, the turbidity measurement in the control was greater than in all the treated by the 96 h of incubation. The percentage inhibition ranged from 0.28 in *L. casei* FF1 to 52.63 in *L. brevis* SG1 during the 96 h incubation period (Table 5). Result of the dry mycelia weight indicated that the bacteriocins had a suppressive effect on the fungal mass of *P. citrinum.* The effect ranged from 2 to 95% in *L. plantarum* EK1 to *L. brevis* SG1, respectively.

Table 1: Source of Lactic Acid Bacteria (LAB) screened for antifungal activity

Isolates	Codes	Sources
Lactobacillus plantarum	EK 1	Eko from maize
Lactobacillus fermentum	EK 2	Eko from maize
Lactococcus lactis	EK 3	Eko from maize
Lactobacillus plantarum	EK 4	Eko from maize
Pediococcus acidilactici	EK 5	Eko from maize
Streptococcus cremoris	IR 1	Iru from locust bean
Lactobacillus casei	IR 2	Iru from locust bean
Lactobacillus casei	FF 1	Raw fufu from cassava
Lactobacillus plantarum	FF 2	Raw fufu from cassava
Lactobacillus reuteri	FF 3	Raw fufu from cassava
Lactobacillus fermentum	FF 4	Raw fufu from cassava
Lactobacillus reuteri	CF 1	Cooked fufu from cassava
Lactobacillus brevis	SG 1	Ogi from sorghum
Lactobacillus plantarum	SG 2	Ogi from sorghum
Lactococcus lactis	SG 3	Ogi from sorghum
Lactobacillus casei	SG 4	Ogi from sorghum
Lactobacillus fermentum	SG 5	Ogi from sorghum
Lactobacillus acidophilus	SG 6	Ogi from sorghum
Lactobacillus bulgaricus	SG 7	Ogi from sorghum
Leuconostoc mesenterioides	SG 8	Ogi from sorghum
Leuconostoc lactis	SG 9	Ogi from sorghum
Lactobacillus fermentum	SG 10	Ogi from sorghum
Lactobacillus casei	MG 1	Ogi from maize
Lactobacillus delbrueckii	MG 2	Ogi from maize
Lactobacillus plantarum	MG 3	Ogi from maize

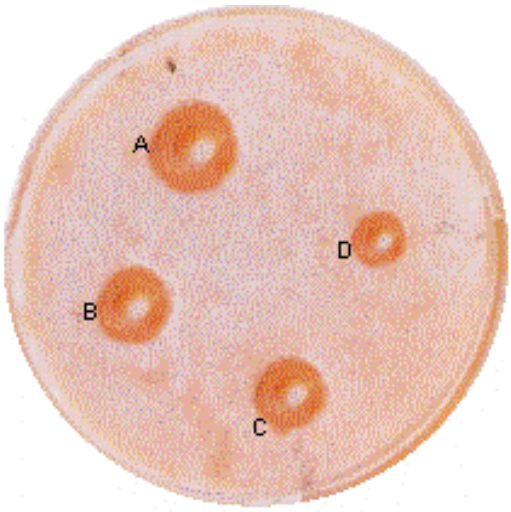

Fig. 1: Effect of pre heating* (at 121°C) on the antifungal activity of *Lactobacillus brevis* SG1 against *Penicillium citrinum*. Exposure period: A: 5 min, B: 10 min, C: 15 min, D: 30 min

Table 2: Inhibition of indicator organisms by cell-free culture supernatants of the lactic acid bacteria isolates*

		Time[b] (days)																		
		A. niger FF2							*P. citrinum* EK1						*A. flavus* EB3					
Microorganism	A	01	02	03	04	07	14	01	02	03	04	07	14	01	02	03	04	07	14
Lactobacillus plantarum EK1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactobacillus fermentum EK2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactococcus lactis EK3	+	6	14	12	nd	nd	nd	nd	6	nd	nd	nd	nd	-	-	-	-	-	-
Lactobacillus plantarum EK4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pediococcus acidilactici EK5	f	13	13	11	10	nd	nd	12	12	10	10	6	nd	nd	4	nd	nd	nd	nd
Streptococcus cremoris IR1	+	4	3	nd	nd	nd	nd	nd	6	4	4	nd	nd	4	3	nd	nd	nd	nd
Lactobacillus casei IR2	f	14	14	14	10	10	6	17	17	17	15	13	8	nd	10	10	7	5	5
Lactobacillus casei FF1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactobacillus plantarum FF2	f	9	9	7	4	4	4	14	14	10	9	6	4	7	9	10	10	nd	nd
Lactobacillus reuteri FF3	+	5	3	nd	nd	nd	nd	6	6	4	nd	nd	nd	-	-	-	-	-	-
Lactobacillus fermentum FF4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactobacillus reuteri CF1	+	nd	10	8	6	nd	nd	nd	10	10	5	nd	nd	-	-	-	-	-	-
Lactobacillus brevis SG1	f	10	12	14	10	10	6	18	18	18	18	18	15	nd	10	10	8	8	5
Lactobacillus plantarum SG2	+	4	3	nd	nd	nd	nd	5	8	nd	nd	nd	nd	-	-	-	-	-	-
Lactococcus lactis SG3	f	15	19	19	16	10	10	12	12	12	10	10	8	14	13	12	12	12	12
Lactobacillus casei SG4	f	12	14	15	12	nd	nd	8	10	12	11	nd	nd	10	11	12	6	nd	nd
Lactobacillus fermentum SG5	+	5	4	nd	nd	nd	nd	nd	4	4	3	nd	nd	-	-	-	-	-	-
Lactobacillus acidophilus SG6	f	nd	8	8	7	5	nd	13	12	10	8	7	5	9	6	6	4	nd	nd
Lactobacillus bulgaricus SG7	+	3	3	nd	nd	nd	nd	-	4	nd	nd	nd	nd	nd	4	3	nd	nd	nd
Leuconostoc mesenterioides SG8	f	13	13	10	8	7	5	14	16	16	14	12	6	5	10	10	9	5	nd
Leuconostoc lactis SG9	f	nd	6	5	4	3	nd	10	10	8	6	nd	nd	7	7	3	nd	nd	nd
Lactobacillus fermentum SG10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactobacillus casei MG1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactobacillus delbrueckii MG2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactobacillus plantarum MG3	+	-	-	-	-	-	-	-	4	2	-	-	-	-	-	-	-	-	-

*Diameter of zone of inhibition (mm). A: Activity, [b]Incubation period (days), -: No inhibition/no inhibition zone, +: Inhibition, f : Fungicidal, nd: Not detectable

Table 3: Inhibitory* effect of bacteriocins of lactic acid bacteria isolates on spore germination of *Penicillium citrinum*

Species	G	Germination (%)[a]	Inhibition (%)[b]
Lactobacillus plantarum EK1	249	83	0.00a
Lactobacillus fermentum EK2	234	78	4.87ab
Lactococcus lactis EK3	216	72	12.20bc
Lactobacillus plantarum EK4	252	84	0.00a
Pediococcus acidilactici EK5	90	30	63.41f
Streptococcus cremoris IR1	204	68	17.07c
Lactobacillus casei IR2	45	15	81.71h
Lactobacillus casei FF1	240	80	2.44ab
Lactobacillus plantarum FF2	78	26	68.29f
Lactobacillus reuteri FF3	62	54	34.15d
Lactobacillus fermentumi FF4	28	76	7.32ab
Lactobacillus reuteri CF1	171	57	30.48d
Lactobacillus brevis SG1	36	12	85.36h
Lactobacillus plantarum SG2	156	52	36.59d
Lactococcus lactis SG3	81	27	67.07f
Lactobacillus casei SG4	120	40	51.27e
Lactobacillus fermentum SG5	207	69	15.85bc
Lactobacillus acidophilus SG6	78	26	68.29f
Lactobacillus bulgaricus SG7	213	71	13.42bc
Leuconostoc mesenteroides SG8	66	22	73.17g
Leuconstoc lactis SG9	108	36	56.10e
Lactobacillus fermentum SG10	246	82	0.00a
Lactobacillus casei MG1	231	77	6.10ab
Lactobacillus delbrueckii MG2	237	79	3.66ab
Lactobacillus plantarum MG3	234	78	4.86ab
Control	246	82	

300 fungal spores were examined. *24 h incubation period, [a](G/300)×100, [b]$1-\sum\frac{(G/300\ \text{treated})}{(G/300\ \text{control})}\times 100$, G: No. of fungal spores that germinated in 300 observations. Means followed by the same letters are not significantly different by DMRT (8 = 0.05)

Table 4: Turbidity measurement* of the growth of *Penicillium citrinum* in the presence of cell-free culture supernatants of lactic acid bacteria isolates

	Absorbance			
	Time (h)			
LAB isolates	24	48	72	96
Lactobacillus plantarum EK1	0.237	0.880	1.146	1.420
Lactobacillus fermentum EK2	0.238	0.856	1.186	1.425
Lactococcus lactis EK3	0.240	0.842	1.074	1.360
Lactobacillus plantarum EK4	0.235	0.882	1.152	1.460
Pediococcocus acidilactici EK5	0.224	0.791	0.992	1.104
Streptococcus cremoris IR1	0.240	0.810	1.032	1.210
Lactobacillus casei IR2	0.200	0.480	0.640	0.724
Lactobacillus casei FF1	0.242	0.850	1.135	1.476
Lactobacillus plantarum FF2	0.198	0.552	0.652	0.714
Lactobacillus reuteri FF3	0.225	0.753	0.994	1.151
Lactobacillus fermentum FF4	0.236	0.860	1.139	1.472
Lactobacillus reuteri CF1	0.235	0.700	0.900	1.086
Lactobacillus brevis SG1	0.196	0.403	0.527	0.701
Lactobacillus plantarum SG2	0.228	0.707	1.010	1.270
Lactococcus lactis SG3	0.207	0.461	0.620	0.756
Lactobacillus casei SG4	0.216	0.552	0.779	1.000
Lactobacillus fermentum SG5	0.226	0.793	1.040	1.216
Lactobacillus acidophilus SG6	0.220	0.602	0.782	0.913
Lactobacillus bulgaricus SG7	0.238	0.846	1.145	1.448
Leuconstoc mesenterioides SG8	0.214	0.550	0.729	0.801
Leuconstoc lactis SG9	0.224	0.601	0.994	0.953
Lactobacillus fermentum SG10	0.235	0.800	1.142	1.410
Lactobacillus casei MG1	0.246	0.850	1.146	1.420
Lactobacillus delbrueckii MG2	0.233	0.860	1.152	1.473
Lactobacillus plantarum MG3	0.234	0.792	1.038	1.278
Control	0.235	0.834	1.146	1.480

*Spectrophotometric reading at 490 nm

Table 5: The percentage inhibition of growth of *Penicillium citrinum* by bacteriocins of the lactic acid bacteria isolates

	Incubation period (h)				
	Inhibition (%)*				
LAB isolates	24	48	72	96	DW**
Lactobacillus plantarum EK1	0.00	0.00	0.00	4.05	2a
Lactobacillus fermentum EK2	0.00	0.00	0.00	3.72	3a
Lactococcus lactis EK3	0.00	0.00	6.28	8.11	7a
Lactobacillus plantarum EK4	0.00	0.00	0.00	1.35	0a
Pediococcus acidilacti EK5	4.69	5.16	13.43	25.41	32c
Streptococcus cremoris IR1	0.00	2.88	9.94	18.24	19b
Lactobacillus casei IR2	14.89	42.45	44.15	51.08	90f
Lactobacillus casei FF1	0.00	0.00	0.96	0.28	0a
Lactobacillus plantarum FF2	15.75	37.41	43.11	51.76	85ef
Lactobacillus reuteri FF3	4.25	9.71	13.43	22.23	30c
Lactobacillus fermentum FF4	0.00	0.00	0.61	0.00	0a
Lactobacillus reuteri CF1	0.00	16.07	21.46	26.62	30c
Lactobacillus brevis SG1	16.59	51.68	54.01	52.63	95f
Lactobacillus plantarum SG2	2.98	7.67	12.65	14.19	16b
Lactococcus lactis SG3	11.91	44.72	45.89	48.92	62d
Lactobacillus casei SG4	8.08	33.81	32.02	32.43	26bc
Lactobacillus fermentum SG5	3.83	4.92	9.90	13.78	15b
Lactobacillus acidophilus SG6	6.38	27.82	31.76	38.31	37c
Lactobacillus bulgaricus SG7	0.00	0.00	0.09	0.00	3a
Leuconostoc mesenteroides SG8	8.51	34.05	36.38	45.88	56.7d
Leuconstoc lactis SG9	4.69	27.82	3.43	35.60	39c
Lactobacillus fermentum SG10	0.85	0.00	0.00	0.40	3a
Lactobacillus casei MG1	0.00	0.00	0.00	4.05	5a
Lactobacillus delbrueckii MG2	0.00	4.08	3.49	4.73	5a
Lactobacillus plantarum MG3	0.43	4.92	9.94	13.65	15b

*Photometric measurement, DW: Inhibition of mycelial dry weight after 5 days incubation. Means followed by the same letter(s) are not significantly different by DMRT (8 = 0.05)

Preliminary Screening for Antifungal Activity

Antifungal activity was highest in the LAB strains from sorghum Ogi (90%) while it was lowest in those obtained from maize Ogi (33%). This demonstrated the high spectra of activity of LAB from sorghum Ogi. The inhibitory zones ranged from 3.0 to 19.0 mm in metabolites of *Lactobacillus bulgaricus* SG7 and *Lactococcus lactis* SG3, respectively against *A. niger* FF2 after 24 h incubation. Inhibitory zones of 18 and 17 mm, respectively were recorded in *P. citrinum* culture when charged against *Lactobacillus brevis* SG1 and *Lactobacillus casei* IR2 , while 2 mm zone was produced against the same fungus by *L. plantarum* MG3 after 72 h incubation. *P. citrinum* EK1 was the best indicator organism as it was sensitive to all the 17 LAB cells after 72 h incubation. This was followed by *A. niger* FF2 and *A. flavus* EB3 (Table 2). Inhibitory effect of some of the LAB strains on *P. citrinum* using the line method is evidenced in Fig. 2.

Nine of the 17 strains exhibited a high fungicidal activity, as there was no reversion of the delay in growth caused by these strains before and after 7 days incubation. Majority (67%) of the 9 strains were obtained from sorghum Ogi while 11% each were from Eko, Iru and Fufu, respectively. The AF effect of the LAB isolates was further examined on the spore germination and growth of *P. citrinum* being the most susceptible fungus.

Inhibition of Spore Germination of *P. citrinum*

The inhibitory effects on spore germination ranged from 2.44% in *L. casei* FF1 to 85.36% in *L. brevis* SG1 (Table 3). The reductive effect was significant as a total of 48% of the LAB isolates exhibited at least 30% inhibition on spore germination of *P. citrinum*. Generally, the

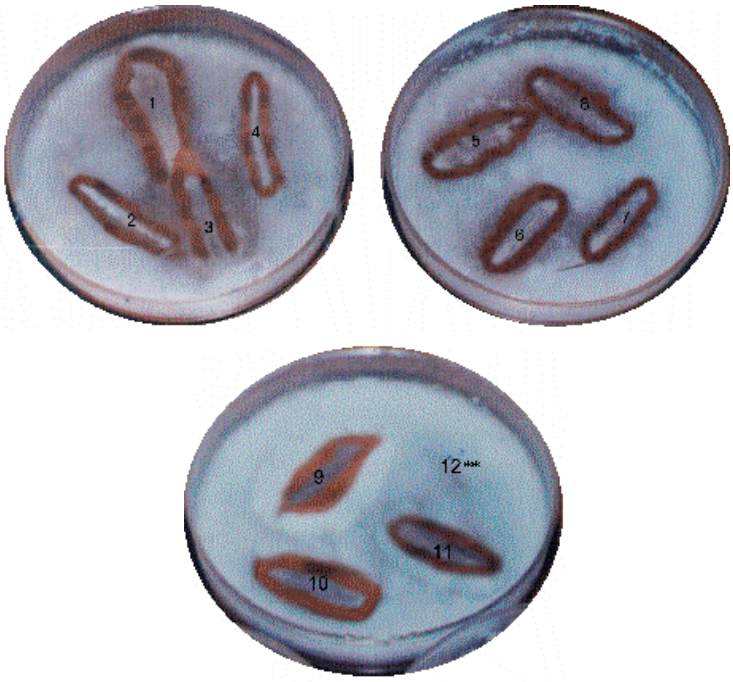

Fig. 2: Antifungal activity of lactic acid bacteria* on the growth of *Penicillium citrinum*. *The organisms are: 1: *Lactobacillus brevis* SG1, 2: *L. casei* IR2, 3: *Leuconosoc mesenteroides* SG8, 4: *L. casei* SG4, 5: *Lactococcus lactis* SG3, 6: *L. fermentum* SG6, 7: *L. plantarum* FF2, 8: *Pediococcus acidilactici* EK5, 9: *Leuconocstoc lactis* SG9, 10: *L. reuteri* CF1, 11: *Streptococcus cremoris* IR1 and 12**: Lactic acid

spores were sensitive to the CFNS of the 17 LAB that possessed AF activity. Germination of the spores after 24 h incubation was high (82%) but reduced to 12, 15 and 22% when treated with bacteriocin from *L. brevis* SG1, *L. casei* IR2 and *Leuconostoc mesenterioides* SG8, respectively. However, there was no 100% germination of the spores in the fungus. Odigie (2000) reported that only a few spores formed by any fungus ever germinated. There was a significant difference in the effect of the different LAB strains on spores germination of *P. citrinum* ($p<0.05$).

Inhibition of Fungal Growth

There was a gradual increase in the absorbance (OD_{490}) with time thus indicating an increased turbidity by the growth of the fungus. The increase in turbidity of the control in comparison with the test fungi indicates a higher mycelia growth (Table 4). The absorbance ranged from 0.704 to 1.473 in the samples treated, with *L. delbrueckii* MG2 having the highest while *L. brevis* SG1 recorded the least

The percent inhibition was very low or undetectable by the 24th h but increased progressively till the 96th h, indicating that the AF activity increased with time. The bacteriocins of the LAB strains caused a reduction on the mycelia mass of *P. citrinum* and the effect of the different LAB strains differed significantly ($p<0.05$) (Table 5).

DISCUSSION

The highest number of isolates recorded in Ogi may be due to its having the highest pH (4.3) (Data not shown) as most LAB are acidophiles. High occurrence of bacteriocinogenic LAB in sorghum Ogi had been previously reported by Adebayo and Aderiye (2007). The higher prevalence of rod-shaped LAB in this study corroborated the study of Ogunbanwo *et al.* (2004), who reported that the genus *Lactobacillaceae* commonly predominate during food fermentation. This is because they are the most aciduric of all LAB.

Preliminary screening of the lactic acid bacteria for antifungal activity demonstrated that the LAB strains were able to produce antimicrobial substances with antifungal activites. This is interesting because bacteriocins from LAB cells are generally known to mainly inhibit bacterial strains (Nes *et al*., 1996). The antagonistic action was produced by catalase-treated, neutralized, cell-free culture filtrates (CFNS), indicating that the antifungal activity was not due to the action of organic acids or hydrogen-peroxide produced by these LAB strains. Previous study showed that the antagonistic activity exhibited by the LAB strains was completely destroyed by treatment with proteolytic enzymes (Adebayo and Aderiye, 2008). The antifungal effect therefore could be attributed to the production of bacteriocins by the LAB strains.

Inhibitory zones were observed within 24 h of incubation period, whereas Vanne *et al.* (2000) reported inhibitory activity on test fungi (*Penicillium* sp.) after 48 h. This tends to suggest the high potency of these antifungal (AF) compounds. The increase in the inhibition zone with time indicated that the effect of the AF compounds increased with time. The reduction or reversion of the delay in growth by the AF compounds from some of the LAB cells after 96 h suggests that these compounds could be fungistatic or that the fungi cells became insensitive to the AF activities of the inhibitory compounds with time.

Whereas *L. plantarum* isolated from Eko and maize Ogi, respectively showed no AF activity against *P. citrinum*., *L. plantarum* obtained from sorghum Ogi displayed a high AF activity against the fungus, suggesting that these organisms (*L. plantarum*) may belong to different strains. It also suggests that the AF activity is strain-dependent rather than species-dependent.

The pattern of sensitivity of *P. citrinum* to the LAB strains in both solid and liquid media appeared to be similar. For instance, all the LAB strains that produced high inhibition zone (Ø≥10 mm) against *P. citrinum* in the agar well bioassay also inhibited the germination of its spores by at least 51% in the liquid media assay. Also, treatment with CFNS from *L. plantarum* EK4 and *L. fermentum* SG10 did not produce any inhibition in the fungus in both bioassays, indicating that the bioassays were comparable and also confirming the reliability of the results.

Some of the LAB strains, *Lactobacillus fermentum* EK2, *L. casei* MG1 and *L. delbrueckii* MG2 that produced no inhibition of growth of *P. citrinum* on the agar displayed 4.87, 4.86 and 6.10% inhibition, respectively against the fungus in liquid media, suggesting that bioassay in liquid media was more sensitive than on the agar media. This could be due to a greater diffusion of the AF compounds in the liquid medium than on the agar medium and that contact with the spores was greater in a liquid medium. Again, the antifungal activity appeared to be strain rather than species dependent. For instance, while strains of *L. plantarum* EK1, EK4 and MG3 showed no AF activity, *L. plantarum* FF2 possessed a higher AF activity against spore germination of *P. citrinum*. The low (%) inhibition of fungal growth by the 24th h may be attributable to the fact that the spores had just started to germinate; hence not much difference could be detected between the control and the treated.

While the inhibition (%) of spore germination ranged from 2.44 to 85.36 by the 24th h, the inhibition (%) of fungal growth was only from 2.98 to 16.59 at the same period, suggesting the superiority of the former over the latter. This is expected because spore germination is the first stage of growth and many of the factors that influence vegetative growth might influence spore germination the more. Although, the turbidity measurement was to assess growth, absorbance after 24 h could be used as a measure of spore germination since the spores germinated within this period. The final growth by the 96th h depended on the initial number of spores that germinated. The ability of the LAB strains to inhibit reproductive structures tends to potentiate their use to prevent fungal spoilage of food, as the infectious fungi need to germinate before colonizing foods.

Whereas the agar-well diffusion assay indicated that only 17 of the 25 LAB strains possessed AF activity by the 96th h, the photometric assay indicated that CFNS of 24 of the LAB strains had a reductive effect on the fungal growth, indicating the higher sensitivity of the spectroscopic assay. Again this might be attributable to the direct contact of the spores and the AF compounds in the liquid medium rather than on the solid agar where the compounds had to diffuse through a medium. There was no significant difference in the effect produced by *L. casei* IR2 and *L. brevis* SG1 on mycelia day mass, indicating that either of the two organisms could be used to produce the same effect on *P. citrinum.*

An inhibition level greater than 25% was observed in 10 of the fungi toxic LAB strains (17). Vanne *et al.* (2000), who reported that growth of toxigenic storage fungi of cereals can be restricted by LAB *in vitro* achieved an inhibition level of 40% in 22 of 104 combinations of LAB and *P. verrucosum.* Niku-Paavola *et al.* (1999) also reported on the AF effects of *L. plantarum* and concluded that inhibition was due to a combined effect of lactic acid and antimicrobial substances produced by the bacteria. While Lavermicocca *et al.* (2000) attributed the AF activities of LAB used in their study to the effect of phenyl lactic acid; Magnusson *et al.* (2003) observed that inhibition was due to the action of hydroxylated fatty acids produced by the LAB. Ghildiyal and Pandley (2008) also reported that antibiosis, myco-parasitism and competition for nutrients are the mechanisms involved in biological control.

This study demonstrated that germination of fungal spores and fungal growth were significantly reduced by the presence of the LAB cells and/or their cell-free filtrates, thus indicating the propensity of the use of these AF substances in biocontrol. Germination of the fungal spores was more sensitive to the bacteriocins than fungal growth. Inhibition of fungal growth and spore germination was more detectable in liquid cultures than on solid media. The results of all the bioassays were comparable confirming the effectiveness of the antifungal compounds.

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