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ANTAGONISTIC ACTIVITY OF LACTIC ACID BACTERIA AGAINST MYCO-DETERIOGENS OF *AGIDI* AND *FUFU*, TWO AFRICAN FERMENTED FOODS

[*1]Dike K. S., [2]Ezekiel C. N., [3]Abiala M.A, [3]Sanni A.I.
[1]Department of Applied Microbiology and Brewing, Nnamdi Azikiwe University, Awka, Nigeria.
[2]Dept of Biosciences and Biotechnology, Babcock University, PMB 21244, Ikeja, Lagos 100 001, Nigeria.
[3]Department of Botany and Microbiology, University of Ibadan, Ibadan, Nigeria.

ABSTRACT

The potential of indigenous Lactic acid bacteria (LAB) from intermediate product stages of agidi and fufu processing to exert antagonistic activity against spoilage mould from same foods in vitro was studied. A total of 27 LAB strains belonging to four genera: Lactobacillus, Lactococcus, Pediococcus and Leuconostoc; were recovered from the various fermented cereal gruels and retted cassava. Lactobacillus plantarum, L. brevis and L. fermentum were the predominant species isolated. The myco-deteriogens from deteriorating fufu and agidi samples were Aspergillus niger, A. flavus, A. fumigatus, Rhizopus sp. and Penicillium sp. The LAB strains showed a weak to strong antagonistic activity against the deteriogenic moulds, most of which were significant ($p<0.05$). L. plantarum had the highest antagonistic effect against A. fumigatus and A. flavus while Lactococcus lactis and P. acidilactici exerted the highest mean antagonistic activity against A. niger and Rhizopus sp., respectively. The LAB species produced a weak antagonistic action against Penicillium sp. with no significant difference ($p>0.05$) in their individual effects towards this deteriogenic mould. The test LAB strains produced lactic acid, hydrogen peroxide and diacetyl in the broth in varying concentrations. A weak positive correlation ($r = 0.355$) was recorded between the highest concentration of lactic acid (g/l) produced by each LAB species and the total antagonistic activity against all fungi in contrast to the negative correlation ($r = -0.175$) for the other two metabolites tested.

Keywords: Metabolites; Inhibition; Lactic acid bacteria; Deteriogens; Agidi; Fufu

INTRODUCTION

The Lactic acid bacteria (LAB) are a broad group of gram positive, catalase negative, non sporing rods and cocci, usually non motile that utilizes carbohydrates fermentation to form lactic acid as the major end product (Aguirre and Collins, 1993). They are found in many nutrient rich environments and occur naturally in various food products such as dairy, meat products and vegetables (Carr *et al.,* 2002). They have, by tradition, been established as a natural, consumer and environment friendly way of preserving food. Their preserving effect is mainly due to the reduction of pH through the production of lactic acid. Besides lactic acid, several other antimicrobials are produced during the growth of LAB (Lindgren and Dobrogosz, 1990; Sanni *et al*., 1999; Ogunbanwo *et al*., 2004; Dike and Sanni, 2010).
Moulds and yeasts are important spoilage organisms in different food systems (Fapohunda and Olajuyigbe, 2006; Ezekiel *et al*., 2010). They have been reported to deteriorate food materials by the liberation of extracellular proteins after the invasion of the food. However, there has been a long standing commercial interest in using LAB as a natural food preservative to increase food safety and stability (Daeschel, 1989, 1993). This interest has been masked by the use of artificial preservatives, which has given rise to concerns from consumers, and an increased awareness of the microbiological safety of such foods. Nowadays, consumers favour foods with few chemical preservatives. There is increased interest in the preservation of food through LAB because of their safe association with human fermented foods and feeds coupled to their natural acceptance as GRAS (Generally Regarded as Safe) product for human consumption (Caplice and Fitgerald, 1999). This present work aimed at evaluating the *in vitro* inhibition of deteriogenic moulds by indigenous lactic acid bacteria isolates, a study that may suggest their use as food preservatives against these deteriogens which affect traditional fermented foods. The concentrations of some extracellular metabolites from the LAB isolates were also quantified.

MATERIALS AND METHODS

Samples

Samples of maize (*Zea mays*), sorghum (*Sorghum vulgarae*) and millet (*Elusine caracana*) grains were purchased from Bodija market in Ibadan, Southwestern Nigeria to be used for making cereal gruels. The cereal gruels served as source of LAB alongside retted cassava which was also collected for making *fufu*. Already prepared *agidi* and *fufu* were purchased from various markets in Ibadan metropolis and served as the source for the recovery of spoilage moulds. The retted cassava samples were collected in sterile plastic containers while all other samples were brought into the laboratory in separate clean polyethylene bags for immediate analysis.

Isolation and identification of LAB

LAB were isolated from retted cassava samples and samples of 2 days fermented cereal gruels of white and yellow maize, white and red sorghum, and millet as described by Halm *et al*. (1993). Isolates were identified according to Kandler and Weiss (1986) and Smeath (1986) by cell and colony morphology, Gram staining, catalase test, growth at 15^{o}C and 45^{o}C, spore staining, motility test and other biochemical tests like oxidase, indole production, methyl red, Voges-proskauer, liberation of ammonia from arginine, growth in 4% broth, hydrogen sulphide production, growth at 4% NaCl, casein hydrolysis and carbohydrate fermentation.

Isolation and identification of myco-deteriogens

Mould isolates were obtained by direct plating of randomly excised portions of the food samples (*agidi* and *fufu*) on ¼ strength Potato Dextrose Agar (PDA) amended with 10ml/L chloramphenicol and 5ml/L streptomycin. Inoculated plates were incubated at 25^{o}C for 3–5days after which isolates were purified on PDA. Identification of fungi was by careful study of macro- and micro-characters and comparison with micrographs and descriptions in Domsch and Anderson (1970).

Preparation of LAB inoculum

Each of the lactic acid bacteria was suspended first in sterile skimmed milk for upwards of 18h, taking samples every 3h and determining the state of growth using a Neubauer haemocytometer until they showed steady growth. Thereafter, each isolate was transferred to sterile de Man, Rogosa and Sharpe (MRS) broth in which the steady state was maintained at 10^6 cells/ml.

Inhibition of myco-deteriogens by LAB isolates

The potential of all LAB strains to inhibit deteriogenic moulds isolated from *fufu* and *agidi* were determined by the modified overlay method of Magnusson and Schnurer (2000). Briefly, each LAB isolate was inoculated on a freshly prepared MRS agar plate as 2 cm long inoculum line and incubated at 30^{o}C for 48 h in anaerobic jars. The incubated plates were then overlaid with a thin layer of 1/4 strength agar preparation of PDA containing 1.0 x 10^4 spores/ml fungal spores as determined by counting on a Neubauer haemocytometer. This was set in triplicates for each LAB isolate against each of test fungus and plates were incubated further at 30^{o}C for 4 days. After 4 days the plates were examined for clear zones of inhibition around the line of bacterial growth. The clear zones were scored as: –, indicating no zone of inhibition; +, inhibition zones ≤ 5mm; ++, zone of inhibition of 5mm < x ≤ 10mm; +++, indicating inhibition zones of 10mm < x ≤ 15mm. The scored values were used to generate the mean antagonistic activity of the LAB strains by species.

Quantitative determination of extracellular (cell-free) metabolites

The antimicrobial compounds that characterize LAB were determined quantitatively in seven selected strains that showed high antagonistic potential against the moulds. The test organisms were grown in MRS broth for 48h and centrifuged thereafter at 3000g for 15mins. The amounts of lactic acid, hydrogen peroxide (H_2O_2) and diacetyl produced as metabolic by-products in the broth by the isolates were assayed titremetically according to A.O.A.C. (1990) as reported by Dike and Sanni (2010).

Statistical analysis

The SPSS® 14.0 package was used for all analysis. One way ANOVA and Duncan's multiple range test (DMRT) was used for the separation of means and test of significance at p=0.05 in the antagonistic activity study of the LAB species against each deteriogenic mould. The relationship between the peak concentration of each extracellular metabolite produced and the overall antagonistic effect exerted on the fungi by each LAB species was correlated.

RESULTS

A total of 27 LAB strains belonging to four genera: *Lactobacillus*, *Lactococcus*, *Pediococcus* and *Leuconostoc*; were recovered from the various fermented cereal gruels and retted cassava. The isolates were identified as *Lactobacillus plantarum, L. casei, L. delbrueckii, L. fermentum*, *L. acidophilus*, *Lactococcus lactis, Pediococcus acidilactici* and *Leuconostoc mesenteroides*. The LAB occurred most in the fermented cassava mash (*fufu*) with 63% (17/27) of the isolates being recovered while *L. plantarum*, *L. brevis* and *L. fermentum* were the predominant species isolated (data not shown). The isolated and identified myco-deteriogens from the deteriorating *fufu* and *agidi* samples were *Aspergillus niger*, *A. flavus, A. fumigatus, Rhizopus* sp. and *Penicillium* sp. *A. niger* was the most occurring deteriogenic mould in the two food samples with an incidence of 60 – 65% in each of the two samples while the least distributed mould in *fufu* and *agidi* were *Penicillium* sp. and *A. fumigatus*, respectively (data not shown). *Rhizopus* sp. was not recovered from any *fufu* sample. The *in vitro* antagonistic activity of the LAB strains against the spoilage moulds was reported as mean antagonism (mm) for the LAB species by summarizing the effects of individual strains (Table 1). *Lc. lactis* exerted the highest mean antagonistic effect against *A. niger* although no significant difference ($p>0.05$) in antagonistic effect was seen on this mould by this bacterium and others except *L. fermentum*. *L. plantarum* showed high and significant ($p<0.05$) antagonistic effect against the other two species of deteriogenic *Aspergillus* (*A. fumigatus* and *A. flavus*). Although *P. acidilactici* showed the highest antagonistic activity against *Rhizopus*, there was no significant ($p>0.05$) difference in its effect and that of *L. plantarum*. *P. acidilactici* however, did not show any antagonistic effect on *A. fumigatus* and *A. flavus* while *Leuc. mesenteroides* could not antagonize the growth of *A. flavus* and *Penicillium* sp. *L. casei* and *Lc. lactis* also did not exert any antagonistic effect towards *Rhizopus* sp. On the overall, the LAB species seemed to produce a weak antagonistic action against *Penicillium* sp. with no significant difference ($p>0.05$) in their individual effects towards this deteriogenic mould.The test LAB (7 selected strains) produced lactic acid, H_2O_2 and diacetyl as antimicrobial compounds in varying concentrations within different durations (Fig. 1–3). The highest concentrations of the three extracellular metabolites were produced within 48h. *P. acidilactici* consistently maintained its highest production of the three metabolites at 48h whereas other LAB strains produced the highest concentrations of one or two of the antimicrobials within 36h and the others at 48h. *Leuc. mesenteroides* did not liberate lactic acid in this study until 24h of incubation. *P. acidilactici* produced the highest concentration of lactic acid (12.0g/l) within 48h after which it decreased to 6.2g/l at 72h (Fig. 1) while *L. brevis* was the least producer of this metabolite, producing its highest concentration (5.5g/l) within 36h. The concentrations of H_2O_2 in the cell free extract for each test LAB (Fig. 2) indicate that *L. fermentum* produced the highest concentration (65mg/l) at 48h while *L. casei*, which produced 40mg/l within 36h of incubation, was the least producer in terms of peak production. It was observed that the concentrations of H_2O_2 declined linearly for each tested LAB strain immediately after the peak concentration. For diacetyl (Fig. 3), *L. fermentum* was the best producer liberating 6.5g/l diacetyl as the peak concentration at 48h after which production decreased to 1.9g/l at 72h of incubation. *L. casei* produced the highest concentration of diacetyl (4.0g/l) at 36h. There was a weak positive correlation ($r = 0.355$) between the highest concentration of lactic acid (g/l) produced by each LAB species and the total antagonistic activity against all fungi. Conversely there was a negative correlation ($r = -0.175$) for the peak concentration of hydrogen peroxide and diacetyl from the LAB species as against the antagonistic effect on the fungi.

DISCUSSION

Lactic acid bacteria have been reported to naturally occur in a wide array of traditionally fermented food materials (Oyewole and Odunfa, 1990; Olasupo *et al*., 1997; Ogiehor *et al*., 2005; Padonou *et al*., 2009). The

high incidence of LAB in the fermented cassava mash (*fufu*) (63%) coupled with the occurrence of *L. plantarum*, *L. brevis* and *L. fermentum* as predominant species isolated from the cereal gruels and retted cassava corroborates the findings of Oyewole and Odunfa (1990), Oyewole (1991), Olasupo *et al*. (1997) and Onilude *et al*. (2005). These bacteria serve as starter cultures in the fementation of the foods and their relative incidences vary from food to food depending on type and availability of utilizable nutrient, competition, and type and quantity of metabolic products released, among many other factors. The spoilage mould isolated from spoilt *agidi* and *fufu*; two indigenously fermented foods, have also been reported to occur in fermented foods and beverages by other authors (Corsetti *et al.* 1998; Pitt and Hocking, 1999). The high incidence of *Aspergillus niger* (>60%) in both fermented foods indicates deterioration and is worthy of note since Ezekiel *et al*. (2010) suggested that this mould is capable of initiating serious food deterioration due to its prolific capacity to liberate high quantities of extracellular proteins such as amylases, needed for rapid colonization and breakdown of the complex carbohydrates present in food materials. The question of food invasion and spoilage by moulds has continued to generate a lot of interest and the search for possible food preservatives continues. The antagonistic effect of the LAB strains showed a weak to strong activity against fungal growth and this confirms the earlier works of Gourama and Bullerman (1995), Vanne *et al*. (2000) and Magnusson and Schnurer (2001). From previous data of the antagonistic effect of LAB strains against moulds, *Lactobacillus* especially *L. plantarum*, *Lc. lactis* and *L. casei* have been extensively studied to produce great antagonism. However in this study we report that *L. plantarum*, *Lc. lactis* and *P. acidilactici* exerted the most antagonistic effect on the aspergilli and *Rhizopus*. Roy *et al*. (1996) reported *Lc. lactis* CHD to have antifungal activity against *A. flavus, A. parasiticus* and *Fusarium* sp. while Gourama and Bullerman (1995) and Vanne *et al*. (2000) reported *L. casei* to have significant antifungal activity against toxigenic *Aspergillus* and *Penicillium* sp. Latila *et al*. (2002) also suggested that *L. plantarum* exerted much antagonism towards *Fusarium* during malting of barley while Onilude *et al*. (2005) also documented that *L. plantarum* could antagonize and maximally inhibit the vegetative and sporulative growth of all tested aflatoxigenic aspergilli in their study. All these evidences support our reports although we could not lay hands on any data for *P. acidilactici*. The low antagonistic activity of the LAB strains towards *Penicillium* sp. as compared to their high activity towards other tested fungi may be due to the inherent potential of many species of *Penicillium* to liberate catalase, a H_2O_2-scavenging enzyme. Macarisin *et al*. (2007) reported that *P. digitatum* and *P. expansum* liberated catalase as a first line self defensive mechanism and this enhanced their pathogenicity in invading and destroying citrus fruits. In line with this, we may then suggest that our test *Penicillium* sp. may have liberated this enzyme to cleave H_2O_2 to water and oxygen thereby making it less potent to destroy its cell. However, the highest mean antagonism (4.0±1.73) recorded against this mould by *L. casei* correlates with the reports of Vanne *et al*. (2000) who reported *L. casei* to antagonize significantly *Penicillium* sp. The inhibitory activity of LAB against other bacteria and fungi has been suspected to be due to the extracellular metabolites (lactic acid, diacetyl, H_2O_2, bacteriocins or bactericidal proteins) liberated into the microenvironment by the LAB (Daeschel, 1989; Lindgren and Dobrogosz, 1990; Daeschel, 1993; Ogunbanwo *et al*. 2003a, b), however there has not been any available report as to the which of these metabolites is the most active in each case. This may have been due to the difficulty in different food matrices considered, diverse strains and different growth/assay media utilized. In this study, we found that the highest concentration of lactic acid was produced by *P. acidilactici*, a notable producer of lactic acid while *L. fermentum* liberated the highest concentration of H_2O_2 and diacetyl. However, we tried to correlate the peak concentrations of metabolites produced by each LAB species with the overall antagonistic activity on fungi in an attempt to show the metabolite that may have played the most role in each case of antagonism. Our data which showed a weak positive correlation (r = 0.355) for peak lactic acid concentration (g/l) produced and total antagonistic activity in contrast to the negative correlation (r = -0.175) for the peak concentration of H_2O_2 (mg/l) and diacetyl (g/l) from the LAB species as against the antagonistic effect on the fungi seemed interesting. Although all correlations were low, we could establish that lactic acid contributed more to the antagonism of the fungi by the LAB than H_2O_2 and diacetyl bearing in mind that we did not exhaust all determinations for possible extracellular metabolites from the LAB cultures. In view of this finding and that of Lavermicocca *et al.* (2000), who reported that novel phenyllactic acids produced by *L. plantarum* strain 21B isolated from sourdough exerted high significant *in vitro* antifungal activity against *Fusarium graminearum*, *A. niger*, *A. flavus*, *Monilia sitophila*,

Endomyces fibuliger and various species of *Eurotium* and *Penicillium* in a wheat flour hydrolysate culture, we can suggest that lactic acid from LAB tends to exert more antagonistic effect against fungi *in vitro* than diacetyl and H_2O_2 but this maybe dependent on several other factors such as its concentration in medium, type of medium, presence of other metabolites. Conclusively, this study has elucidated the implication of moulds as spoilage organisms in two African fermented food and the potential of indigenous LAB strains in antagonizing the proliferation of the moulds by the liberation of extracellular metabolites *In vitro*. This study is relevant in the area of food fermentation and preservation. Further work will be targeted towards studying other metabolites from the LAB and their specific involvement in antagonism for industrial purposes.

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Table 1: Mean ± SD* antagonistic activity (mm) of some LAB strains by species against deteriogenic moulds of *agidi* and *fufu*

	LAB strains by species						
Deteriogens	*Lactobacillus casei*	*L. plantarum*	*L. fermentum*	*L. brevis*	*Lactococcus lactis*	*Leuconostoc mesenteroides*	*Pediococcus acidilactici*
A. niger	5.0 ± 4.36^{ab}	7.0 ± 0.71^{a}	1.8 ± 0.45^{b}	4.0 ± 0.71^{ab}	12.5 ± 0.71^{a}	11.0 ± 1.41^{a}	5.0 ± 0.00^{ab}
A. fumigatus	7.0 ± 1.00^{ab}	10.4 ± 2.19^{a}	3.8 ± 2.68^{bc}	1.8 ± 1.1^{c}	6.0 ± 0.00^{b}	3.5 ± 2.12^{bc}	---
A. flavus	6.0 ± 5.20^{b}	9.4 ± 0.89^{a}	5.6 ± 4.27^{b}	8.4 ± 5.13^{a}	1.0 ± 0.71^{c}	---	---
Rhizopus sp.	---	11.4 ± 1.34^{a}	4.6 ± 1.95^{b}	3.8 ± 3.03^{b}	---	3.0 ± 1.41^{b}	11.5 ± 0.71^{a}
Penicillium sp.	4.0 ± 1.73^{a}	1.0 ± 1.00^{a}	2.0 ± 1.41^{a}	3.0 ± 1.73^{a}	2.0 ± 0.00^{a}	---	1.0 ± 0.00^{a}

Means with same alphabet in a row are not significantly different at $p>0.05$.
*SD = standard deviation
---: no inhibition zone

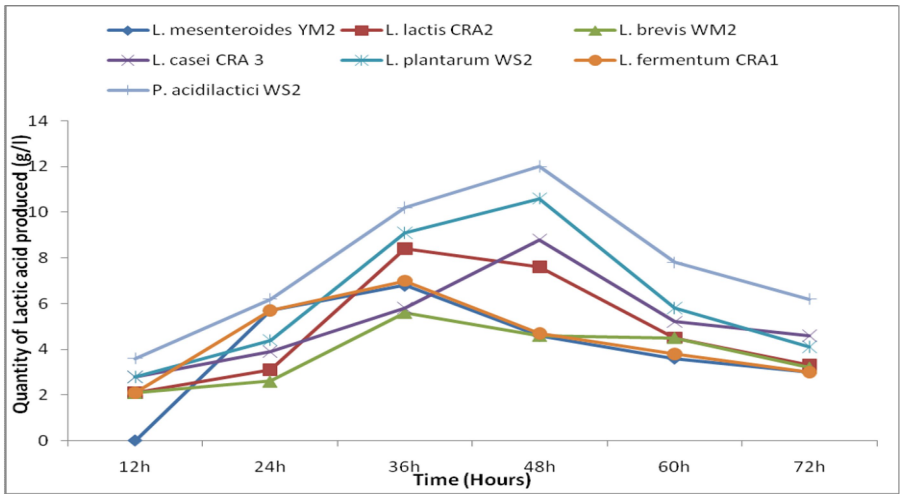

Fig. 1: Lactic acid concentrations produced by LAB isolates in broth cultures at different time intervals

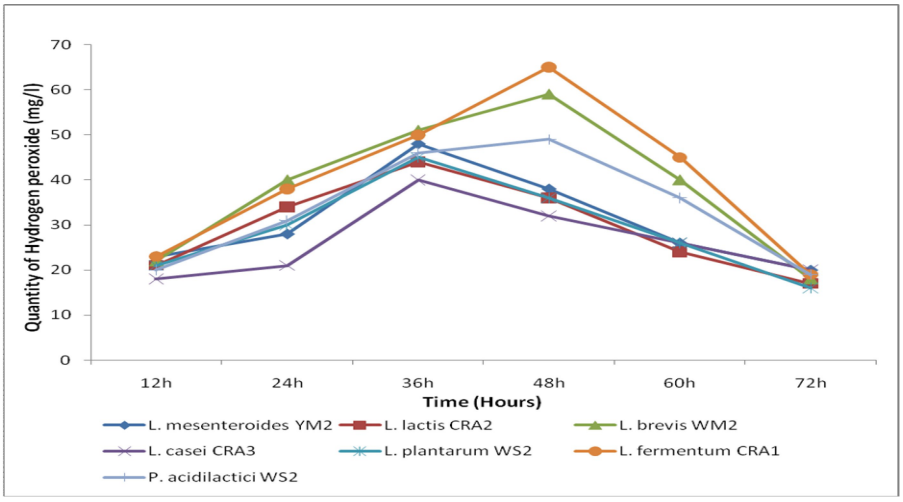

Fig. 2: Hydrogen peroxide concentrations produced by LAB isolates in broth cultures at different time intervals

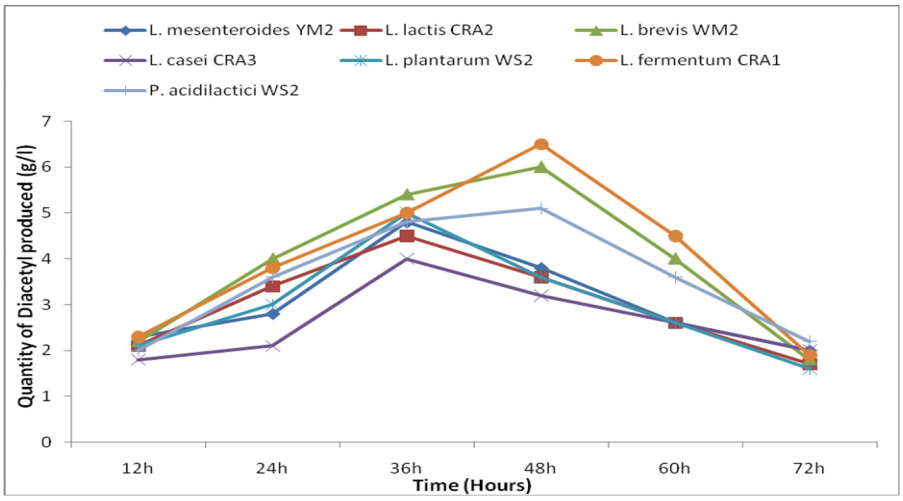

Fig. 3: Diacetyl concentrations produced by LAB isolates in broth cultures at different time intervals