Global Advanced Research Journal of Food Science and Technology (ISSN: 2315-5098) Vol. 2(1) pp. 001-006, February, 2013
Available online http://garj.org/garjfst/index.htm

Full Length Research Paper

Antifungal properties of lactic acid bacteria (LAB) isolated from *Ricinus communis*, *Pentaclethra macrophylla* and Yoghurts

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Accepted 09 January 2012

The antifungal activity of lactic acid bacteria (LAB) isolates from *Ricinus communis* (Ogiri), *Pentaclethra macrophylla* (Ugba) and Yoghurt samples against moulds associated with food spoilage *Aspergillus niger, Aspergillus flavus, Aspergillus fumigatus, Mucor, Penicillium, Rhizopus* and *Aspergillus nidulans* was investigated using agar diffusion technique. *Aspergillus fumigatus* was the most susceptible with inhibition zone diameters ranging from 8mm for *Lactococcus lactis* to 20mm for positive control fluconazole. Pediococcus spp had no antifungal effect on any of the test fungi. None of the LAB isolates had good activity against *Aspergillus flavus* and Rhizopus spp. *Aspergillus niger* was inhibited only by *Lactococcus lactis*. The present study indicates that the LAB isolates if optimized and improved could be used as a natural, food-grade biopreservative agent for management of fungal contamination and food spoilage, thus preventing problems associated with mycotoxins in food and feed products. It is suggested that effective GMP and HACCP application in handling the affairs of food and feed storage/production will no doubt make the use of LAB as preservative a lot easier.

Keywords: Antifungal, Biopreservative, Lactic acid bacteria, Ogiri, Ugba, Yoghurt.

INTRODUCTION

Fungi have a profound biological and economic impact as food spoilage agents, decomposers, plant and animal pathogens. Their ability to grow anywhere, on anything, makes them both beneficial and harmful recyclers of carbon and nitrogen. Fungal spoilage of food and feed is a common and global phenomenon (Pitt and Hocking, 1999). In addition to the negative financial consequences, fungal spoilage of food and feed also poses a serious health concern. Fungal growth on foodstuffs can result in the production of mycotoxins which are known to be toxic to human and animals (Sweeney and Dobson, 1998; Kabak *et al*., 2006).

Fungi have the ability to grow in a wide variety of foods, with different genera showing affinity for particular food types. Fusarium species e.g. are notorious for spoilage of grains and cereal plants in the field. They produce fumonisins, secondary metabolites that are thought to result in immunosuppression as well as carcinogenesis in animals (Chu and Li, 1994; Alves *et al*., 2000; Berek *et al*., 2001). Production of DON by barley-associated *Fusarium culmorum* and *Fusarium graminearum* strains give rise to a phenomenon called gushing (Schwartz *et al*., 1995), which means that toxins may survive the malting process and end up in the finished product.

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Table 1. LAB and fungal isolates from samples

Sample	Microbial isolates
Ogiri	Pediococcus spp, Streptococcus spp, Bacillus spp, *Leuconostoc mesenteroides*, Lactobacillus spp
Ugba	*Lactobacillus salivarius*, *L. plantarum*, Lactobacillus spp, Bacillus spp
Yoghurt	*Lactococcus lactis*, Streptococcus spp, Lactobacillus spp
Arachis hypogara	*Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger, Aspergillus nidulans*
Zea mays	Penicillium spp, Rhizopus spp, *Aspergillus flavus, Penicilium funiculosum, Geotrichum candidum*
Bread	*Sacharomyces cerevisiae*, Mucor spp, Rhizopus spp, *Aspergillus fumigatus, Aspergillus niger*

Postharvest stored cereals are frequently contaminated with Aspergillus and Penicillium species (Filtenborg *et al*., 1996). These moulds are known to produce mycotoxins which may ultimately be carried over into the finished product. For this reason, these organisms constitute a realistic health hazard to the brewing, breakfast cereals and baking industry (Legan, 1993; Araguas *et al*., 2005).

Maize (Zea mays) products are the cheapest and readily available fermented foods for infants and young adults in most tropical countries. It is one of the most widely distributed food plants in the world. They are important energy food rich in carbohydrates and with traces of vitamins, proteins and minerals. Its infection by fungi *Aspergillus flavus*, *A. parasiticus* and *A. nominus* can result in mycotoxin contamination during the growing, harvesting, storage, transporting and processing stages (Achterberg *et al*., 1994; FAO, 2009).

The aflatoxin produced by *Aspergillus flavus* in improperly stored grain is one of the most potent carcinogens yet discovered. Fortunately, other fungi such as Penicillium have been used to develop modern antibiotics and beneficial immunosupresants (Alexopoulos *et al*., 1996). Strains of *Penicillium norcadicum* are commonly isolated from fermented meat products such as cured ham and dairy products such as cheesses and may produce ochratoxin A in these foods (Castella *et al*., 2002)

Dairy products, including milk and cheese, are also susceptible to fungal growth, leading to deterioration, although certain moulds play an important role in cheese production. Species of Penicillium, including *Penicillium commune* and *Penicillium solitum,* have been found to spoil different varieties of cheese (Baslico *et al*., 2001).

With consumer preference for naturally produced foods at an all time high, the need for minimal processing and natural preservation methods is obvious. Lactic acid bacteria (LAB) are naturally occurring in many food systems (Vaughan *et al*., 2001; Tamminen *et al*., 2004; Mitra *et al*., 2005), and have been used for centuries for their fermentation and preservative properties. In particular, their ability to produce antibacterial peptides or bacteriocins has received a lot of scientific attention (Magnusson *et al*., 2003; Cotter *et al*., 2005; Drider *et al*., 2006), while they also sometimes exhibit antifungal activity (Laitila *et al*., 2002; Francesca *et al*., 2002; Dal-Bello *et al*., 2007; Rouse *et al*., 2008, Gerez, 2010, Wulijideligen and Miyamoto, 2011). Several low molecular weight compounds have been isolated with the capacity to eliminate fungal growth either on their own or synergistically, including organic acids, reuterin, fatty acids, proteinaceous compounds and cyclic dipeptides (Cabo *et al*., 2002; Sjogren *et al.,2003*).

Lactic acid bacteria have been used as biopreservatives in food and animal feed, sauerkraut and silage (Messens, 2002). Their preserving effect relates mainly to the formation of organic acids and hydrogen peroxide, competition for nutrient and production of antimicrobial substances formic acid, propionic acid, acetoin and diacetyl (Stiles, 1996; Corsetti, 1998). It is well known fact that increasing amounts of microorganisms are becoming resistant to antimicrobial agents. Fungi are not exception and more species of both human fungal pathogens and spoilage moulds in food and feed systems are becoming resistant. However, yeasts and moulds are not only becoming resistant to antifungals, but also to preservatives such as sorbic acid, benzoic acid, as well as chemical treatment with cleaning compounds (Brul and Coote, 1999).

The general public wants to reduce the use of chemical preservatives in food or feed. Instead, consumers require high quality, preservatives free, safe but mildly processed food with extended shelf life. This is of course not an easy task to solve. In addition, present legislation has restricted the use of some currently accepted preservatives in different foods (Brul and Coote, 1999). Modern concepts to reduce the contamination of products with fungi and mycotoxins will involve the application of biopreservation. In particular, lactic acid bacteria are considered to be appropriate organisms to suppress the growth of other microorganisms including yeasts and moulds.

This work attempt to investigate antifungal activity of LAB from Ogiri, Ugba and Yoghurt samples with a view to contribute to biopreservation.

Table 2. Susceptibility of Fungal isolates to LAB

Fungal isolates	LAB isolates zones of inhibition (mm)								
	Posive control Fluconazol (4mg)	Pediococcus spp	Streptococcus spp	Lactobacillus spp	*L. mesenteroides*	*L. lactis*	*L. plantarum*	*L. salivarius*	Negative control Normal Saline
A. flavus	10	-	-	2	-	-	-	-	-
A. niger	16	-	-	-	-	15	-	3	-
A. fumigatus	20	-	13	15	10	8	-	-	-
Mucor spp	8	-	-	4	-	-	5	-	-
Penicillium spp	15	-	3	-	-	6	-	-	-
Rhizopus spp	6	-	-	-	-	4	-	-	-
A. nidulans	15	-	-	6	-	-	-	-	-

MATERIALS AND METHODS

Sample collection and treatment

Ten samples each of Ogiri and Ugba were purchased from food vendors in Ekeonunwa and Ihiagwa markets in Owerri. Two each of five different brands of yoghurt were purchased from street vendors and shopping malls in Owerri metropolis. The samples of yoghurt were shaken vigorously in stirrer (Jenway, UK) to mix before sample analysis. Ten fold serial dilutions were carried out 10^{-1} to 10^{-6} for all the samples of Ogiri, Ugba and Yoghurts. Aliquot 0.2ml of the dilutions were spread onto duplicate sterile plates of MRS agar and M17 agar (both from Biolab, Hungary) for isolation of lactobacillus.

Thirty samples comprising of ten samples of different brands of breads obtained from vendors in Owerri, Nekede, and Ihiagwa, ten samples each of *Zea mays* and *Arachis hypogaea* from different vendors in four markets in Owerri, Ihiagwa and Obinze were assayed for fungal contaminants. Two cups approximate 200g of *Zea mays* and *Arachis hypogaea* samples was purchased from the vendors, the samples were rinsed out in sterile distilled water for fungal isolation. The bread was left exposed in the laboratory for two weeks before being used for fungi isolation. Sample homogenate from the maize (*Zea mays)*, ground nut (*Arachis hypogaea*) and bread were serially diluted to 10^{-6} and cultured by spread plate technique on Sabouraud dextrose agar (SDA) (Oxoid, England) to which Penicilin and Streptomycin had been incorporated for fungi isolation

All the culture plates except SDA were incubated anaerobically using Oxoid anaerobic jar plus gas pack at 37ºC for 24 to 72hrs. SDA plates were incubated at 28±2ºC for 3-5 days. At the expiration of incubation period, the plates were examined for microbial growth. Typical and characteristic colonies on MRS and M17 agar were purified by repeated subculture on M17 agar to obtain pure culture for further characterization. Discrete colonies on SDA were isolated and purified by repeated subculturing. Pure cultures were stored on slants for further characterization.

Identification of isolates

Standard microbiological procedures were adopted for the identification of probable LAB isolates. Preliminary identification was based on cultural characteristics, Gram staining reaction, and biochemical tests such as catalase, citrate utilization, indole, oxidase, motility, temperature tolerance, growth on concentrations of NaCl, methylene blue, haemolysis on blood agar, sugar fermentation tests, reaction on TSI, gelatin liquefaction, starch hydrolysis, nitrate reduction, methyl red, Voges-proskaur test. Further characterization was by the methods as described by Jolt *et al*., (1994).

Fungal isolates was identified based on their macroscopic cultural characteristics on slide culture and microscopic characteristics with reference to standard

identification atlas and keys (De Hoog *et al*., 2000; Tsuneo, 2010).

Antifungal assay

Preparation of LAB isolates

Overnight broth culture of LAB was standardized to $1.5x10^8$ cells using 0.5 McFarland turbidity standards. MRS agar was uniformly seeded with standardized culture and incubated for 48 hrs at 37ºC

Preparation of fungi inocula

Fungal spores on SDA slants were inoculated onto fresh agar plates and incubated at 28±2ºC until sporulation. The cultures where then taken with sterile needle and introduced into sterile test tubes and standardized to turbidity equivalent to $1.5x\ 10^8$ cells by McFarland standard 0.5. SDA agar was uniformly seeded and incubated at 28±2ºC for growth.

Assay of LAB for antifungal activity

Assay was carried out in two ways: Using a cock borer an agar disk was removed from SDA fungal culture and discarded, this was replaced with LAB culture from MRS. Plates were examined for zone of inhibition after 24 to 96 hrs incubation at 30ºC. Into 5ml of standardized fungal culture in test tube was added 5ml of standardized LAB culture. Incubation was for 24 to 96 hrs and subculture was made onto SDA plus Penicillin and Streptomycin for fungal count after 24 to 96 hrs incubation at 30ºC.

RESULTS

The LAB and fungal isolates from the samples are as shown in Table 1. The prevalence of specific LAB appears to be dependent on the type of sample. *Aspergillus niger* and *Aspergillus flavus* are the predominant contaminants in *Arachis hypogaea, Zea mays* and Bread.

Table 2 reveals the susceptibility of fungal isolates to LAB. *Lactococcus lactis* and a species lactobacillus had inhibitory effects on *Aspergillus niger, Aspergillus fumigatus* and a species of Mucor and Rhizopus. The positive control Fluconazole had inhibitory effects on all the fungal isolates though, with varying zones of inhibition. All the LAB isolates showed inhibitory effects on *Aspergillus fumigatus* except however, for *Lactobacillus salivarius*.

There was a reduction in counts of *Aspergillus fumigatus* and *Aspergillus niger* from 1.5×10^8 cfu/ml to 1.2×10^7 and 1.5×10^6 cfu/ml by Lactobacillus spp and *Lactococcus lactis*. The positive control Fluconazole reduced fungal counts fron 1.5×10^8 to 1.0×10^4, 1.2×10^7 and 1.2×10^6 cfu/ml for *Aspergillus fumigatus, Aspergillus niger* and *Aspergillus nidulans* respectively.

DISCUSSION

Fungi are known to be major cause of spoilage of food and feed products. Farm crops, post harvest stored grains and food of animal origins are frequently contaminated/spoiled by fungal species resulting in huge economic loses. Some of these fungal contaminants are known to produce deleterious mycotoxins which may ultimately be carried over into the finished product and constitute health hazard to man and livestock (Legan, 1993; Chu and Li, 1994; Schwartz *et al*., 1995; Filtenborg *et a*., 1996; Sweeney and Dobson, 1998; Pitt and Hocking, 1999; Alves *et al;* 2000; Berek *et al*., 2001; Araguas *et al*., 2005; Kabak *et al*., 2006). The presence of LAB in Ogiri, Ugba and Yogurt samples is in tandem with the presence of these organisms in fermented products. LAB has the property of producing lactic acid via fermentation of carbohydrates (Odunfa, 1985; Odunfa and Oyeyiola, 1985; Odunfa, 1986; Jay, 2000). All the LAB that was isolated have previously been reported in association with fermented foods and have been used as probiotics (Reid *et al*., 2005; Ammor, 2007; Ouoba *et al.,* 2008; Hoveyda *et al*., 2009). LAB are known to occur naturally in foods or added as pure cultures to various food products. They are considered to be harmless or even to have an advantage for human health as probiotics. LAB have a generally recognized as safe (GRAS) affirmation and have through tradition, been established as a natural consumer and environmental friendly way to preserve food and feed products (Stiles, 1996; Carr *et al*., 2002).

The isolation of fungal spp in *Arachis hypogaea, Zea mays* and Bread corroborate the understanding that fungi are common environmental contaminants and major spoilage organisms of food and feed products (Bullerman,1977; Bonestroo *et al*., 1993; Filtenborg *et al*, 1996; Corsett *et al*, 1998; Pitt and Hocking, 1999; Mari *et al*., 2003; Casey and Dobson, 2004; Kinay *et al*., 2005; Tournas *et al*., 2006).

Some of the LAB isolates specifically Lactobacillus spp and *Lactococcus lactis*, had inhibitory effects on some fungal contaminants with *Aspergillus fumigatus* being the most susceptible. Some fungi have been reported to be sensitive to the normal by-products of LAB metabolism (Batish *et al*, 1989; Piard and Desmazaeud, 1992; Collins, 2004; Dal-Bello *et al*., 2007; Rouse *et al*., 2008; Gerez, 2010; Wulijideligen and Miyamoto, 2011). Although the inhibitory activity of LAB isolates in this study is not comparable to that obtained by Francesca *et al*. (2002), it confirms the prospect of LAB as a potential

antifungal agent in control of fungal contamination and spoilage of food and feed products.

Optimization and improvement of the antifungal potential of LAB for its use in food and feed preservation holds the key to meeting the general demand by consumers to reduce the use of chemical preservatives and additives in food and feed and the production of high quality, preservative free, safe but mildly processed food with extended shelf life. Effective GMP and HACCP application in handling the affairs of food and feed storage/production will no doubt make the use of LAB as preservative a lot easier.

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