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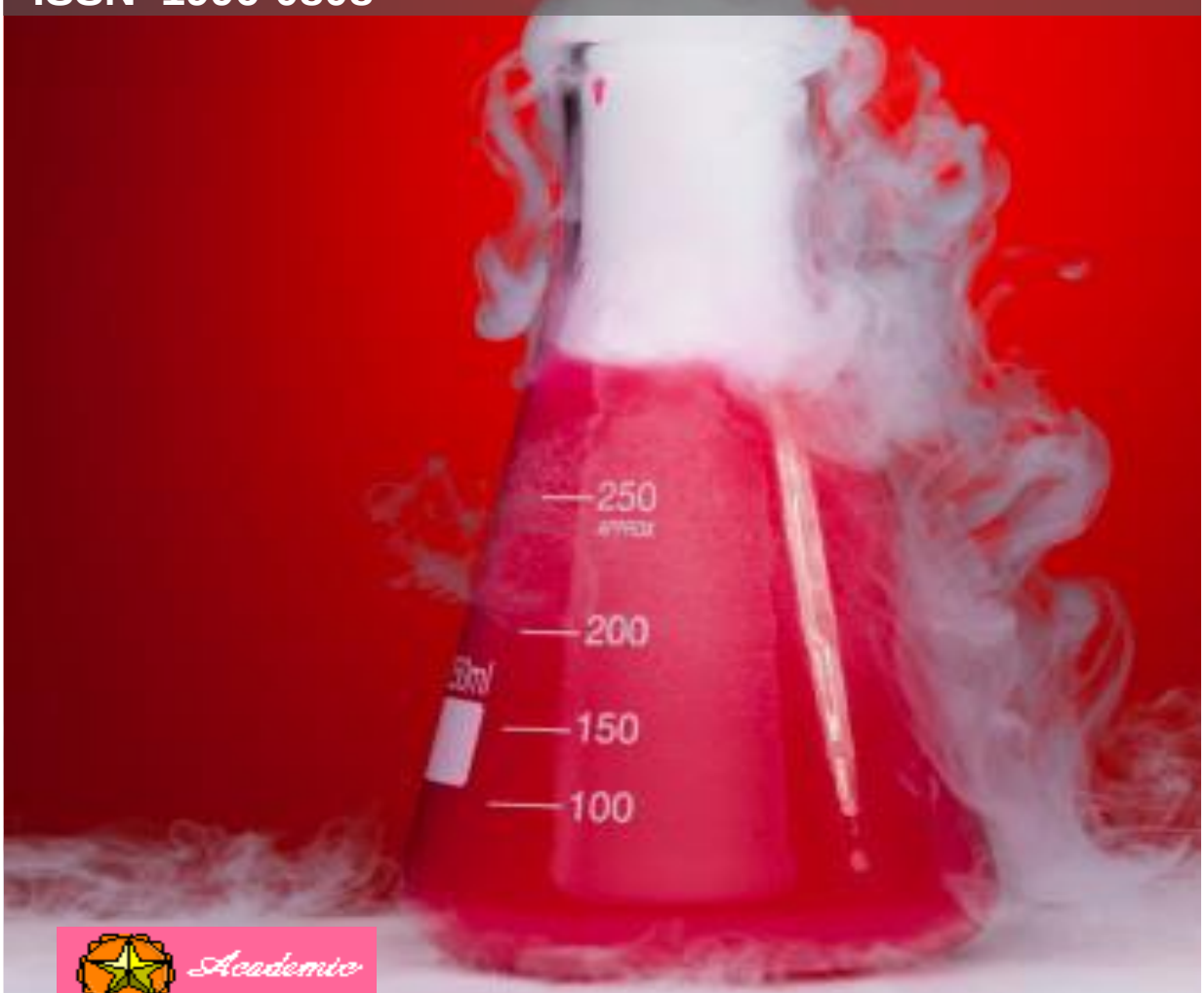

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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603.

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African Journal of Microbiology Research

Full Length Research Paper

Assessment of fungal pathogens associated with orange spoilage

Oviasogie, F. E.[1], Ogofure, A. G.[1*], Beshiru, A.[1], Ode, J. N.[1] and Omeje F. I.[2]

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Citrus sinensis **also known as sweet orange is the most popular of the citrus fruits. It is widely cultivated in most regions of the world possessing a rich source of vitamin C, flavonoids, phenolic compounds and pectin. This research was conducted to investigate the assessments of fungal pathogens associated with orange fruit spoilage sold in five markets in Benin metropolis and the possible public health implications. Some pathogenic fungal species were isolated from all five markets used in this study.** ***Aspergillus*** **species had the highest frequency and distribution from all sampling points (80%).** ***Alternaria*** **and** ***Saccharomyces cerevisiae*** **had the least occurrence from all sampling points (40% apiece).** ***Candida, Mucor, Penicillium*** **and** ***Rhizopus*** **had 60% occurrences, respectively.** ***Candida tropicalis*** **and species of** ***Rhizopus***, ***Penicillium***, ***Aspergillus***, ***Alternaria***, **and** ***Mucor*** **produced same symptoms and signs as observed in the original spoilt orange fruits before isolation. All fungal isolates were able to re-infect the healthy orange fruits with the exception of** ***Alternaria*** **species and** ***Saccharomyces cerevisiae*** **which were not able to grow and produce spoilage condition on the inoculated healthy orange fruits after five days.** ***Aspergillus*** **spp. are known to produce several toxic metabolites, like aflatoxins and ochratoxins, which are very important toxins worldwide because of the hazard it poses to human and animal health.**

Key word: Pathogenicity test, *Aspergillus* sp., *Alternaria* sp., pathogens.

INTRODUCTION

Fruits and vegetables are very important and have high dietary and nutritional qualities. Consumption of fruit and vegetable products has dramatically increased by more than 30% during the past few decades (Barth et al., 2009). They are good sources of nutrients for growth, repair and control of body processes as most of them contain sugar, vitamins, mineral elements and small quantities of protein and oil (Zubbair, 2009). *Citrus sinensis,* also known as sweet orange, is the most popular of the citrus fruits. It is widely cultivated in most

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regions of the world (Muhammad et al., 2013). Oranges form a rich source of vitamin C, flavonoids, phenolic compounds and pectins. The main flavonoids found in citrus species are hesperidine, narirutin, naringin and eriocitrin (Ghasemia et al., 2009). Just one orange provides 116% of the daily requirement for vitamin C. Vitamin C is the primary water-soluble antioxidant, which prevents free radical generation in the body and damage to the tissues in the aqueous environment both inside and outside cells (Milind and Diev, 2012). Drinking of orange juice without salt and sugar is associated with reduced severity of inflammatory conditions, like asthma, osteo-arthritis, and rheumatoid arthritis. Vitamin C is also necessary for the proper functioning of immune system. Vitamin C is good for preventing cold, cough and recurrent ear infections (Guanieri et al., 2007). These losses are due to many factors, among which post-harvest fungal diseases are considered as principal cause. Sweet orange are vulnerable to post-harvest diseases. It was observed in previous studies, that the extent of damage varied from 29.9 to 43.8% in sweet orange and 25.5 to 36.8% in acid lime (Reddy et al., 2008). Studies have shown that oranges have been found to protect the moderate consumer against cardiovascular diseases (Milind and Diev, 2012), possess anti-carcinogenic properties (Tanaka et al., 1997), reduce the risk of kidney stones (Honow et al., 2003), possess anti-ulcer properties (Simon et al., 2003), antianxiety effect (Fsaturi et al., 2010), anti-typhoid activity (Vivek et al., 2010), antibacterial activity (Milind and Diev, 2012) and antifungal activities (Neeta and Abhishek, 2008) amongst the many medicinal uses.

The improper handling, packaging, storage and transportation may result in decay and growth of microorganisms, which become activated because of the changing physiological state of the fruits and vegetables (Wilson et al., 1991). Fruit, due to their low pH, higher moisture content and nutrient composition are very susceptible to attack by pathogenic fungi, which in addition to causing rots, may also make them unfit for consumption by producing mycotoxins (Moss, 2002). The principle of spread of fungal infection in fruits supports that a single infected orange fruit can be the source of infection to other orange fruits during storage and on transit (Jay, 2003). Soil-infesting fungi and bacteria that cause loss of fleshy tissue typically infect plants at the time of or just before harvesting. Infection may occur, however, during post-harvest handling or storage. Common air molds such as *Penicillium* species may gain entry into the susceptible tissue and cause loss during packaging. *Penicillium digitatum* and *Penicillium italicum* causes green and blue mold diseases respectively which are universal post-harvest diseases of citrus. The extensive spore production by these pathogens ensures its presence wherever fruit was handled, including field, packing house, equipment, de-greening, storage rooms, transit containers and market place (Ismail and Zhang, 2004).

The aim of this study was to isolate and identify fungal pathogens associated with orange spoilage in Benin City metropolis, Edo state, Nigeria, using five markets as case study.

MATERIALS AND METHODS

Collection of samples

Healthy, viable orange fruits were purchased from different markets in Benin City, Edo State. The orange fruits were transported in sterile polyethylene bags to the laboratory for analysis.

Physical examination of sample

The physical examinations of spoiled or diseased oranges were identified using the method of (Balali et al. (1995) where various types of spoilt oranges were selected including those that were mechanically wounded or bruised, with purplish to dark brown rot, blue rot, green rot as well as those with black lesions on them.

Preparation of culture medium

Potato dextrose agar was used for isolation of fungi from the Citrus fruits and for the preparation of pure cultures. The medium was prepared from commercially produced dehydrated medium following the manufacturer's instruction. Thirty-nine (39) grams of Potato Dextrose agar powder was dissolved in 1 L of distilled water in a sterile conical flask covered with cotton wool and aluminium foil paper. It was mixed thoroughly and autoclaved at 121°C for 15 min under a pressure of 15 pounds per square inch (15lb/inch^2). The medium was cooled after autoclaving to 50°C and then dispensed aseptically into sterile Petri dishes. Streptomycin (0.3%w/v) was added to the medium to prevent the growth of bacteria.

Isolation of fungi

Two hundred (200) fruits samples were washed and sterilized with 70% ethanol. The borderline between healthy and infected tissue of surface fruits was cut with sterile razor blade. The cut portion of the lesion was disinfected with ethanol of 70% concentration for 2 min. These were then rinsed in three different changes of distilled water. Each excised portion of the infected part showing lesions were plated in Potato Dextrose Agar plates containing streptomycin (30 mg/l) to prevent the growth of bacteria. The plates were incubated at room temperature (28°C) for 72 h.

Identification of fungal Isolates

The fungi isolates were identified on the basis of macro-morphological and micro-morphological characteristics. The morphological characteristics which include colony growth and colour, presence or absence of aerial mycelium, presence or absence of wrinkles and furrows, presence or absence of pigmentation amongst others were observed under the microscope (Thiyam and Sharma, 2013; Barnett and Hunter, 1972) and recorded. In all cases, a drop of lactophenol blue stain was placed

on a clean grease-free sterilized glass slide after which a sterile inoculating wire loop was used to pick the mycelium unto the glass slide from the mold culture. The mycelium was then spread evenly on the slide. Teasing was done to separate the mycelium in order to get a homogenous mixture and the mixture was then covered with cover slips gently and then allowed to stay for some seconds before observing with the microscope under ×40 magnification lens. The microscope examination of actively growing mold was on the basis of structures bearing spores, presence or absence of septa.

Pathogenicity test

Pathogenicity test was carried out as described by Baiyewu et al. (2007) and Chukwuka et al. (2010) where each of the fungal isolates were tested on healthy fruits for its ability to induce spoilage. The methods by these authors are outlined below:

a) Clean mature healthy fruits were washed with tap water and rinsed with distilled water after which they were surface sterilized with 75% ethanol.

b) A sterile 4 mm cork borer was used to make holes in each of the fruits.

c) A colony of fungi isolate (from each pure culture) was used to inoculate the fruits and the core of the fruits were replaced.

d) The point of inoculation was sealed with petroleum jelly to prevent contamination.

e) Controls of orange fruits were wounded with the sterilized cork borer but not inoculated.

f) The inoculated fruits and the controls were placed in clean polyethylene bag (one fruit per bag) each moistened with wet balls of absorbent cotton wool to create a humid environment and incubated at 30 ± 1°C for 5 days.

e) After 72 h, the inoculated fruits were observed for symptom development.

f) The causal agents were re-isolated from the infected orange fruit and compared with the original isolates. This experiment was replicated three times.

RESULTS

The Table 1 shows the occurrence and distribution of each fungal isolates from the five sampling points. *Aspergillus niger*, *Mucor* species and *Rhizopus* species were fungal species isolated from New Benin market. *A. niger*, *P. chrysogenum*, *R. stolonifer*, *Alternaria*, *C. tropicalis* and *Mucor* species were fungal isolates from sweet orange from Oba market. Fungal isolates such as *A. niger*, *Alternaria* species, *R. stolonifer*, *C. tropicalis* and *Saccharomyces cerevisiae* were identified from sweet oranges obtained from Uselu market while Satanna market sweet oranges had fungal isolates such as *Penicillium digitatum*, *C. tropicalis* and *Mucor* species.

Table 2 shows the percentage occurrence and distribution of the fungal isolates from all sampling market points. While *Aspergillus* species had the highest percentage occurrence (80%), *Alternaria* species had the lowest percentage occurrence (40%).

Table 3 reveals the pathogenicity test on fresh healthy citrus fruit samples. From day 0 to day 5, *Alternaria* species and *Saccharomyces cerevisae* were not able to grow on the sample. However, from day 1 to day 5, *Rhizopus*, *Penicillium* and *Aspergillus* species were able to grow with similar growth characteristic features to the original diseased sample. More so, *Mucor* and *Candida* species were also able to grow with similar growth characteristic features to the original diseased samples from day 2 to day 5.

Table 4 describes the spoilage pattern on sweet orange (*Citrus sinensis*) produced by isolated fungal species. Overall, the fungal isolates include *A. niger*. *C. tropicalis*, *R. stolonifer*, *P*. chrysogenum, *P*. *digitatum* and *M*. species.

DISCUSSION

It is estimated that about 20-25% of the harvested orange fruits can be deteriorated by pathogens during post-harvest handling even in developed countries (Droby, 2006; Zhu, 2006). In developing countries, postharvest losses are often more severe due to inadequate storage and transportation facilities. Fungal fruits infection may occur during the growing season, harvesting, handling, transport and post-harvest storage and marketing conditions, or after purchasing by the consumer.

Orange fruits contain high levels of sugars and nutrients element and their low pH values make them particularly desirable to fungal decay (Singh and Sharma, 2007).

The seven fruit spoilage fungi were isolated from the two hundred (200) orange fruit samples and identified as *Aspergillus* species, *Mucor* species, *Penicillium*, *Rhizopus*, *C. tropicalis, S. cerevisiae* and *Alternaria* species. *Aspergillus* spp. are widespread among all examined spoilage fruits with the highest percentage occurrence of 80% from all sampling points. Fourty orange fruit were sampled in each market point. *Aspergillus* species, *Penicillium* species, *Mucor* species and *Rhizopus* species were able to cause spoilage on re-infection with healthy fruits, while *Alternaria* species showed no growth. Bukar et al. (2009) reported that *Aspergillus* species, *Mucor*, *Penicillium* species and *Rhizopus* sp, which are the same genus with those isolated from orange fruits in this study, as responsible for the soft rots of orange fruits in Nigeria. The spoilt sweet oranges sampled from the different markets in Benin City were found to be massively infected by different species of fungi. This is similar to the findings of Bukar et al. (2009) who reported that diseased oranges sampled from Na'ibawa yan Lemu Market in Kano were found to be massively infected with six genera of fungi namely *Fusarium, Aspergillus*, *Candida, Rhizopus, Penicillium* and *Mucor*. The occurrence of these organisms may be attributed to their ability to produce resistant spores, as reported by Hocking (2006) that “Aspergilli generally grow at higher temperatures or lower

Table 1. Occurrence and distribution of the fungal isolates from the sampling market points.

Fungal isolates	Ekiosa	New Benin	Oba market	Uselu market	Satanna market
Rhizopus species	-	+	+	+	-
Penicillium species	+	-	+	-	+
Aspergillus species	+	+	+	+	-
Alternaria species	-	-	+	+	-
Mucor species	-	+	+	-	+
Candida tropicalis	+	-	-	+	+
Saccaromyces cerevisiae	-	-	+	+	-

+, represent presence; -, represent absence.

water activities than Penicillia and they usually grow more rapidly than Penicillia, although they take longer to sporulate, and produce spores which often are more resistant to light and chemicals".

Akintobi et al. (2011) reported that *Aspergillus flavus, A. niger, Fusarium solani, Penicillium digitatum, R. stolonifer* and yeasts were found in fruits sold in major markets in Ibadan, Oyo State, South Western Nigeria. *P. digitatum*, *R. stolonifer*, and *A. niger* were found to be associated with spoilage or deterioration of orange fruits in Ibadan (Akintobi et al., 2011). This could be due to the presence of their spores, which in turn releases toxins into the oranges or even releases enzymes which could contribute to the deterioration of the oranges. Samples from Oba market had the highest occurrence of fungal isolates. This might be due to the high population density as the market is located at the heart of the city where virtually anyone from all classes can be found to carry out one economic activities. Sweet oranges from Satanna market had the least occurrence of fungal isolates and this could be as a result of improved personal hygiene of handlers or good storage methods. Taking the population of and size of the market into consideration, it is far smaller than Oba market, Uselu market and more likely, Ekiosa market. In conformity with this research, Effiuvwevwere (2000) reported that contamination of fruits and vegetables by fungi could be as a result of poor handling practices in food supply chain, damage inflicted on fruits at time of harvest creating a route for spores of pathogenic fungus, poor storage condition, distribution, marketing practices and transportation. Different spoilage types were observed on re-inflection of healthy oranges with pure isolate of fungi species. This could be as a result of the ability of the fungi species to survive in the oranges especially when the environmental conditions are favourable, producing spores, toxins and enzymes. This is similar to the findings of Bukar et al. (2009) who reported that different spoilage types were observed when the healthy oranges were re-inoculated with the pure isolates of the pathogens. Some however, did not cause spoilage on re-inoculation. *Aspergillus* species, *Penicillium*, *C. tropicalis*, *Mucor* species and *Rhizopus* species were the fungi that caused spoilage of the sweet oranges in this study. This is in conformity with Bukar et al. (2009) who revealed that *Aspergillus* species, *Penicillium* species, *Mucor* species and *Fusarium* species were the fungal that were able to cause re-infection in the healthy oranges after the pathogenicity tests.

Several fruit spoilage fungi from different region has been isolated and identified. *A. niger* and *C. tropicalis* were found associated with deterioration of orange; this is in line with the work of Nijis et al. (1997) who reported that *Aspergillus* species is the predominant organism associated with the spoilage of orange. The isolation of *R. stolonifer* and *Mucor* species from orange confirmed the studies of Bukar et al. (2009) who reported that *Mucor* species and *Rhizopus stolonifer* are responsible for the spoilage of orange.

Similarly, Voysey (2011) reported that *Alternaria* sp. causes black rot in citrus fruits, *Aspergillus* species causes brown rot of citrus fruits and pineapple, *Penicillium* species causes blue and green mould rots of citrus fruits, apples, grapes, pears and also brown rot of pineapple, *Aspergillus* species and *R. stolonifer* causes watery, soft rot of apples, pears, stone fruits and grapes, *Geotrichum* species causes sour rot of citrus fruits, *Trichoderma* species causes cocoa-brown to green rot of citrus fruits. The principle of spread of fungal infection in fruits supports that a single infected orange can be the source of infection to other oranges during storage and on transit (Jay, 2003).

The presence of the fungi or their resistant spores is most likely to have originated from the farms where the fruits were harvested and some from the stores due to horizontal contamination by the already spoilt fruits as Jay (2003) observed that most spoilage organisms may be present on fruits and vegetables from the farm, during harvest operations, and this may result in post-harvest contamination and spoilage of these fruits and vegetables. The present and subsequent spoilage due to these fungi, if not checked could lead to serious economic loss and possible health hazards when these

Table 2. Percentage of occurrence and distribution of the fungal isolates from all sampling market points.

Fungal isolates	Percentage occurrence (%)
Aspergillus species	80
Penicillium species	60
Mucor species	60
Rhizopus species	60
Candida tropicalis	60
Saccharomyces cerevisiae	40
Alternaria species	40

Table 3. Pathogenicity test on fresh healthy citrus fruit samples.

Fungal isolates	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
Rhizopus species	-	+	+	+	+	+
Penicillium species	-	+	+	+	+	+
Aspergillus species	-	+	+	+	+	+
Alternaria species	-	-	-	-	-	-
Mucor species	-	-	+	+	+	+
Candida tropicalis	-	-	+	+	+	+
Saccaromyces cerevisiae	-	-	-	-	-	-

+ = Isolates grow with a similar growth characteristic features to the original diseased samples; - = Isolates not able to grow on the sample.

Table 4. Spoilage pattern on sweet orange (*Citrus sinensis*) produced by isolated fungal species.

Fungal isolates	Spoilage pattern produced
Aspergillus niger	Dark brown discoloration, sunken spots, fruits become spongy with gas production
Candida tropicalis	Fruit becoming spongy with gas production, sunken spots
Rhizopus stolonifer	Watery, soft rot wrinkled appearance with depression and yellowish in color
Penicillium chrysogenum	Wrinkled appearances, pale green-blue, exuding bright yellow pigment into the medium
Penicillium digitatum	Wrinkled appearances with sunken spots and ;live green in color
Mucor species	Whitish mycelia growth with a cream white color

fruits are consumed. Generally, spoilage fungi are considered toxigenic or pathogenic. Toxigenic fungi have been isolated from spoilt fruits (Stinson et al., 1981). During storage at room temperature, some moulds may produce mycotoxins (Tournas and Katsoudas, 2005). Pathogenic fungi, on the other hand, could cause infections or allergies (Monso, 2004). *Aspergillus* spp. are known to produce several toxic metabolites, such as malformins, naphthopyrones (Frisvad and Samson, 1991; Pitt and Hocking, 1997) and they can produce Ochratoxins (OTA), a mycotoxin which is a very important toxin worldwide because of the hazard it poses to human and animal health (Peraica et al., 1999; Petzinger and Weidenbach, 2002) thus extra care should be taken during personnel handling of these fruits; such as harvesting, cleaning, sorting, packaging, transport and storage.

The high prevalence of some fungi demand that appropriate control measures against infection, should be employed if farmers expect good performance of their produce. The fruits used in this study are not cultivated in the city but are transported to from distant villages in locally woven baskets and sacks under weather conditions that encourage the incubation of these contaminating microorganisms. It is therefore important that both the farmer who harvests the fruits into bags for transportation, the marketers and consumers take

necessary precaution in preventing contamination and also try to create an environment that will not encourage the growth or multiplication of microorganisms.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Antifungal activity of *Citharexylum quadrangulare* Jacq. extracts against phytopathogenic fungi

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Antifungal activity of *Citharexylum quadrangulare* Jacq., an exotic tree introduced in Tunisia many years ago, was evaluated. Organic extracts using hexane, ethyl acetate and methanol solvents together with aqueous extracts tested at different concentrations were prepared from different organs (roots, stems, leaves and flowers). The fungitoxic activity for all extracts was evaluated against five phypathogenic fungi (*Fusarium culmorum, F. graminearum, Aspergillus flavus*, *Aspergillus niger* and *A. fumigatus*). All parameters were used in a principal components analysis (PCA) and a hierarchical clusters analysis (HCA). This study concludes that some aqueous and organic extracts of *C. quadrangulare* could be potential sources of natural fungicides to protect crops from fungal diseases.

Key words: Antifungal, biofungicides, *Citharexylum quadrangulare* Jacq., extracts, safe environment.

INTRODUCTION

A great diversity of fungi cause plant diseases; nearly all major groups are involved. Fungal infections are one of the major problems associated with our daily life. Generally, phytopathogenic fungi are controlled by synthetic fungicides; however, the use of these is increasingly restricted due to the harmful effects of pesticides on human health and the environment (Dellavalle et al., 2011). Hence, there is a great demand for novel antifungals belonging to a wide range of structural classes, selectively acting on new targets with fewer side effects, particularly from plant origin. The increased development of resistance against frequently used antimicrobial compounds by the microorganisms necessitates the discovery of new antimicrobial compounds in plant extracts.

Many plants synthesize substances that are useful for controlling the growth of microorganisms and plants are a possible source of antimicrobial agents non phytotoxic, systemic and biodegradable (Adesina et al., 2000; Ouedraogo et al, 2013; Sumathy et al., 2014; Kumari et al., 2015; Gond et al., 2015; Martins et al., 2015).

Recent studies showed that crude extracts of various plant species such as *Coronopus didymus* (L.) Sm., *Datura metel* L., *Solanum indicum* L., *Azadirachta indica* A. Juss., *Oxalis latifolia* Kunth and *Jatropha curcas* L. were very effective in the management of

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Table 1. Aqueous and organic extracts of *Citharexylum quadrangulare* used for antifungal tests and abbreviation.

Collection date	Collection locality	Plant part sampled	Plant source	Fungi
02/04/2010	MenzelBouzelfa	Fruits	Citrus	*Aspergillus flavus*
02/04/2010	Jendouba	Leaves	Wheat	*A. niger*
02/04/2010	Nefza	Leaves	Wheat	*A. fumigatus*
23/05/2007	Beja	Roots and stems	Wheat	*Fusarium culmorum*
23/05/2007	Beja	Roots and stems	Wheat	*F. graminearum*

phytopathogens namely *Sclerotium rolfsii* Sacc., *Fusarium oxysporum* f. sp. *lycopersici*, *Aspergillus flavus* and *Alternaria alternata* (Iqbal and Javaid, 2012; Jabeen et al., 2014; Anil and Raj, 2015; Abd El-Ghany et al., 2015). Researchers have also isolated many potential antifungal compounds from plants such as *β*-amyrin from *Melia azedarach* L. (Jabeen et al., 2011), tow flavonoids 7-O-glucoside and (-)-epi catechin from *Azadirachta indica* (Kanwal et al., 2011), and methyl gallate from *Melaphis chinensis* (Kuo et al., 2015). Gond et al. (2015) reported that lipopeptides secreted by bacterial endophytes naturally occurring in many maize varieties inhibit pathogens and for Martins et al. (2015) fungicidal effects proved to be dependent on the occurrence of phenolic compounds in tested extracts.

Citharexylum quadrangulare Jacq. (syn. *Citharexylum spinosum* L. and *Citharexylum fruticosum*) (Verbenaceae) (Wagner et al., 1999) is native to Caribbean (Turner and Wasson, 1997), was introduced in Tunisia many years ago and is cultivated along the roadsides and in gardens. This tree possesses medicinal properties and is useful for the treatment of various ailments. A decoction of young twigs was used for children thrush and bark decoction for treating colds (Lachman-White et al., 1992). The leaves are used as a source of an anti-allergic substance and as an alternative medicine in hepatic disorders (Balazs et al., 2006). *C. quadrangulare* was used in combination with other plants as anthelmintic (Lans, 2007). Studies concerning the antimicrobial activity of *C. quadrangulare* are not yet available except the work recently published by Ae and Patcharee (2014) where authors reported that flowers extracts and flower oil have an interesting antibacterial activity against eight microorganisms

So, the aim of this present study was to determine the antifungal potentialities of *C. quadrangulare* aqueous and organic extracts against five phytopathogenic fungi (*Fusarium culmorum*, *F. graminearum*, *Aspergillus flavus*, *A. niger* and *A. fumigatus*) causing serious damage in agriculture, resulting in losses of crop yield and quality.

MATERIALS AND METHODS

Plant material

C. quadrangulare different organs (roots, stems, leaves and flowers) were collected in the garden of the High Institute of Biotechnology of Monastir (latitude 35º46'0''N, longitude 10º59'0''E, coastal region, East of Tunisia, with a sub humid climate). A voucher specimen (CQV 12) was deposited at the Herbarium of the Laboratory of Botanic in the Institute. Roots were cleaned with tap water, and all the plant parts were air-dried in a shaded area at ambient temperature. Dried material was grounded into a powder using a Wiley mill and stored at 4ºC until use.

Preparation of aqueous and organic extracts

Two hundred grams of powder from each dried plant part were separately extracted by soaking in 100 mL distilled water at ambient temperature for 24 h to give a concentration of 2 g dry tissue per mL. The 16 crude aqueous extracts (Table 1) were filtered through a double layered muslin cloth followed by Whatman no. 1 filter paper and then passed through 0.22 µm micro-filter pore size to remove bacteria. Filtrates were preserved at 4°C. Aqueous extracts were used freshly within a week.

Sequential extraction was carried out in organic solvents with rising polarity: hexane, ethyl acetate and methanol. One hundred grams of powder were immersed in the appropriate solvent for 7 days at room temperature. The 12 organic extracts (Table 1) were evaporated to dryness under reduced pressure in a rotary evaporator at 45°C, to remove the solvent. After determination of the yield the extracts were stored at 4°C until use. Control was used with distilled water and methanol without organic extract.

Fungal isolates

Fungi used in this study were collected from various localities in Tunisia. Each fungus, the host and the plant part from which it was isolated, the locality and the date of collection are listed in Table 2. All fungal isolates were identified (Boughalleb et al., 2006; 2008) and samples of each fungus were deposited in the collection bank at the Plant Pathology Laboratory (High Institute of Agronomy of Chott-Meriem, Tunisia). Fungal isolates were maintained on potato dextrose agar (PDA, Difco Laboratories, Inc., Detroit, MI, USA), stored at room temperature and sub-cultured once a month (Deans and Svoboda, 1990) when needed.

Antifungal bioassays

The antifungal activity was determined according to the poisoned food technique of Grover and Moore (1962). PDA media with 0.25, 0.5, 1 and 2 g/mL of each aqueous extract were prepared. On the other hand each organic extract was dissolved in methanol and added in PDA medium at the concentration of 1 mg/mL before solidification. PDA medium was without aqueous extract and this amended only with methanol served as controls. Fifteen ml of the each medium was poured into Petri plate (9 cm diameter) and

Table 2. Fungal isolates used to test the antifungal activity of *Citharexylum quadrangulare*

Organic extracts	Abbreviation
Root, shoot, leaves, flowers hexane extracts	Rhex, Shex, Lhex, Flhex
Root, shoot, leaves flowers ethyl acetate extracts	Reac, Seac,Leac,Fleac
Root, shoot, leaves flowers methanol extract	Rmet, Smet, Lmet, Flmet
Aqueous extracts	Abbreviation
Aqueous root extracts at 0.25, 0.5, 1, 2 g/ml	AR0.25, AR0.5, AR1, AR2
Aqueous shoot extracts at 0.25, 0.5, 1, 2 g/ml	AS0.25, AS0.5, AS1, AS2
Aqueous leaves extracts at 0.25, 0.5, 1, 2 g/ml	AL0.25, AL0.5, AL1, AL2
Aqueous flowers extracts at 0.25, 0.5, 1, 2 g/ml	AFl0.25, AFl0.5, AFl1, AFl2

allowed to solidify. Then a disc (5 mm) of 7-days-old culture of each tested fungus was taken with a pre-sterilized cork borer and placed upside down on the centre of the dish. The Petri plates were incubated at 25±2°C in the dark for 7 days. The extension diameter (mm) of mycelia from the centre to the sides of the dishes was measured at 24 h intervals for seven days. The fungi toxicity in the aqueous and organic extracts in terms of percentage of mycelia growth inhibition (% MGI) of each fungus was calculated by using the formula of Jabeen and Javaid (2008):

% MGI = dc - dt/dc x 100

Where, dc = Average increase in mycelia growth in control, dt = Average increase in mycelia growth in treatment (aqueous and organic extract).

Statistical analysis

Percentages of mycelia growth inhibition (%MGI) of each fungus were transformed using arcsin-square root (arcsine √x) and subjected to conformity with assumptions of normality for analysis of variance (ANOVA) using SPSS 12.0, for Windows program. The significance of the differences between means was determined at $p < 0.05$ using *Duncans's* multiple range test. We evaluated whether the type of extract (Table 1) (or group of extracts) was useful in reflecting its fungitoxic effect. The %MGI reported with all extracts tested were subjected to Principal Components Analysis (PCA) and Hierarchical Cluster Analysis (HCA) using SPSS 12.0 software (SPSS Inc. Chacago, IL, USA).

RESULTS

The percentages of the mycelia growth inhibition of each fungus were used for the PCA and the HCA (data not given) based on the *Euclidean* distance between groups. Two groups of extracts (1 and 2), identified by the fungus or group of fungus with which they correlate were identified, and data are reported in Table 3. Groups 1 and 2 clearly stand out forming separate groups in the PCA.

Group 1 (Reac, Fleac, Seac)

This group of extracts correlates with the percentages of mycelia growth inhibition for four out of the five fungi tested. In fact, the three extracts inhibited the growth of *A. flavus*, *A. niger* and *A. fumigatus* from 40.38 to 68.6% and to a lesser degree of *F. culmorum* mycelia (19.55-37.18%).

Group 2

The group 2 includes all the 25 other extracts, divided in 2 subgroups 2A and 2B. The subgroup 2A is divided into subgroups 2Aa and 2Ab and 2B into subgroups 2Ba and 2Bb. The later (2Bb) still divided into subgroups 2Bb1 and 2Bb2, and the 2Bb2 yet subdivided in 2 subgroups 2Bb21 and 2Bb22. Extracts from subgroup 2Aa (Smet, AS1, AS2) inhibited *A. niger* at percentages varying from 43.3 to 50.9%. Aqueous extract from stems at 1 mg/ml (AS1) inhibited *A. flavus* at 22.8% and AS1 and AS2 inhibited *F. graminearum* at 24.04%. *A. fumigatus* and *F. culmorum* were not susceptible to those extracts (0 - 10.3%). Those from the subgroup 2Ab (Shex, Lhex, Flhex) were highly positively correlated with the %MGI of *A. niger* (43.6 - 53.8%), and of *F. fumigatus* (39.1 - 50.6%), contrariwise, *A. flavus* and *F. graminearum* were not inhibited. In contact with the two extracts, AR1 and Leac, from the subgroup 2Ba, the %MGI of *A. flavus* were from 63.5 to 65.4%, however, %MGI of *F. culmorum* weakly correlated with those extracts (14.4 to 12.8%). Leaves ethyl acetate extract (Leac) inhibited also *A. fumigatus* (38.5%). *A. niger* and *F. graminearum* were no or weakly inhibited (4.2% for *F. graminearum* with AR1). Subgroup 2Bb1 with 7 aqueous extracts (AR0.25, AR0.5, AL0.5, AL2, AS0.25, AFl, AFl2) correlated with the %MGI of *A. flavus*, *F. graminearum* and *F. culmorum*, but were opposed to the inhibition of *A. niger* (0%) and *A. fumigatus* (0%). Percentages of MGI of *A. flavus* and of *F. graminearum* varied from 14.1 to 49.0% and from 25.9 to 48.4%, respectively. For *A. flavus* the best inhibition was obtained with AL0.5 and AR0.25 and for *F. gramineaum* with AR0.5. *A. niger* was inhibited only with AR0.25 (28.8%). *F. culmorum* was less affected by the 7 extracts (6.4 - 19.9%); extract AS0.25 have no effect on the mycelia growth of this fungus. Subgroup 2Bb2 was

Table 3. Percentages of mycelia growth inhibition in the aqueous and organic extracts (%MGI). The table was established according the Principal Component Analysis and the Hierarchical Clusters Analysis. Organic extracts were tested at 1 mg/ml and aqueous extracts at 0.25, 0.5, 1, 2 g/ml.

Groups/subgroups					*Aq/Org extracts	Mean % of mycelia growth inhibition				
						A. flavus	*A. niger*	*A. fumigatus*	*F. culmorum*	*F. graminearum*
Group 1					Seac	45.8±4.9f	42.6±9.3e	50.3±5.4g	19.6±1.9e-g	1.6±3.9a
					Fleac	68.6±3.6h	51.9±8.1gh	55.8±6.9h	37.2±6.0i	0.0±0.0a
					Reac	57.1±4.5g	49.4±8.2fgh	40.4±7.6f	29.8±7.1h	0.0±0.0a
	2A	2Aa			Smet	0.0±0.0a	46.8±9.2efg	0.0±0.0a	10.3±8.3bc	0.0±0.0a
					AS2	0.0±0.0a	50.9±1.1gh	0.0±0.0a	0.0±0.0a	24.0±9.6c
					AS1	22.8±5.9c-e	43.3±5.0ef	0.0±0.0a	0.0±0.0a	12.2±5.5c
		2Ab			Flhex	0.0±0.0a	53.8±7.1h	47.8±7.8g	6.4±7.3ab	0.0±0.0a
					Lhex	0.0±0.0a	43.6±9.9ef	39.1±4.2f	14.1±4.5c-f	0.0±0.0a
					Shex	0.0±0.0a	53.5±7.8h	50.6±4.2g	13.8±2.6c-f	0.0±0.0a
		2Ba			AR1	65,4±4.2h	00.0±0.0a	0.0±0.0a	14.4±1.1c-f	4.2±7.1a
					Leac	63.5±7.6h	00.0±0.0a	38.5±9.1f	12.8±5.9b-e	0.0±0.0a
			2Bb1		AR0.25	47,5±5.3f	28.8±6.3d	0.0±0.0a	11.5±6.2bc	25.9±9.9c
					AR0.5	17.0±9.3bc	0.0±0.0a	0.0±0.0a	11.2±8.9bc	48.4±9.5f
					AL0.5	49.0±2.0f	0.0±0.0a	0.0±0.0a	14.7±3.8c-f	36.9±8.3e
					AFl1	21.5±9.0cd	0.0±0.0a	0.0±0.0a	15.4±3.8c-f	35.9±9.7e
Group 2					AL2	21.1±7.9cd	0.0±0.0a	0.0±0.0a	19.9±2.3fg	28.2±7.7cd
					AFl2	14.1±8.1b	9.9±9.6b	0.0±0.0a	6.4±8.2ab	33.9±9.2de
					AS0.25	28.2±9.0e	0.0±0.0a	0.0±0.0a	0.0±0.0a	26.3±9.4c
	2B				AFl0.25	0.0±0.0a	22.8±4.3c	13.8±5.1b	13.1±5.4b-f	24.0±6.9c
		2Bb			AFl0.5	0.0±0.0a	0.0±0.0a	21.8±6.2c	22.4±9.2g	20.5±8.1c
					Flmet	0.0±0.0a	0.0±0.0a	20.8±4.6c	16.0±2.3c-g	11.5±4.7b
				2Bb22	Lmet	0.0±0.0a	0.0±0.0a	29.5±8.5e	12.5±5.7b-d	0.0±0.0a
					Rmet	0.0±0.0a	0.0±0.0a	49.7±7.0g	12.5±3.6b-d	0.0±0.0a
			2Bb2		AL1	0.0±0.0a	0.0±0.0a	0.0±0.0a	19.2±3.6d-g	0.0±0.0a
					AL0.25	24.7±9.9de	0.0±0.0a	0.0±0.0a	13.5±4.7c-f	0.0±0.0a
					AR2	27.6±8.6de	0.0±0.0a	0.0±0.0a	16.3±3.2c-g	0.0±0.0a
					AS0.5	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a
				2Bb21	Rhex	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a

*Aq. Aqueous; org. organic; for abbreviation of extracts see Table 1. Means ± SE followed by different letters differ significantly at $p < 5\%$, as established by *Duncan's* test.

distributed in 2Bb21 and 2Bb22. Extracts from the former group (Rhex, AS0.5) did not inhibit all tested fungi. The subgroup 2Bb22 was formed by AFl0.25, AFl0.5, Flmet, Lmet, Rmet, AL0.25, AL1, and AR2 extracts. The first five extracts showed no effect on the development of *A. flavus* and *A. niger* mycelia, except AFl0.25 extract (22.8% against *A. niger*). *A. fumigatus, F. culmorum* and *F. graminearum* were inhibited (11.5 - 49.7%) by those extracts except Lmet and Rmet extracts (against *F. graminearum*). The highest percent of inhibition was exhibited with Rmet extract against *F. fumigatus* (49.7%). AL1, AL0.25, AR2 were ineffective against *A. fumigatus, A. niger* and *F. graminearum* but inhibited *F. culmorum* (13.5 - 19.2%). The extracts AL0.25 and AR2 were revealed to be able to reduce the mycelia growth of *A. flavus* (24.7, 27.6%, respectively). However, the extract AL1, did not inhibit this fungus.

DISCUSSION

Biocontrol is the safest and economical method of controlling plant pathogens by using extracts of different plant parts (Shafique et al., 2011; Kumari et al., 2015; Gond et al., 2015; Martins et al., 2015). The results of this conceptual study clearly reflect that *C. quadrangulare* has inherent ability to induce fungitoxic effects on the *in vitro* growth of the tested fungi species. According to the previously mentioned results, almost organic and aqueous

extracts (AR0.25, 0.5, 1; AL0.5; AS1,2; AFL1,2; Reac, Seac, Leac, Fleac, Shex, Lhex, Flhex, Smet and Rmet) inhibited at least one fungus growth (%MGI >40%). The percentage of *A. flavus* mycelia growth inhibition was higher than 60 with Fleac, Leac and AR1. However, the relative intensity of the antifungal effect varies with the target fungus, as well as the origin, type and concentration of the extract. Fungicidal effects proved to be dependent on the extracts concentration (Martins et al., 2015). The differences recorded between extracts in this fungitoxic test is likely due to the solubility of the active compound(s) in water or to the presence of inhibitors to the fungitoxic principle as noted by Amadioha (2001) and Okigbo and Ogbonnaya (2006).

Data of this study shows that *Aspergillus* species were the most susceptible to tested extracts and *A. niger* was the most affected, depending on the extract and its concentration. Susceptibility of *A. flavus*, *A. niger* and *A. fumigatus* to plant extracts has been previously also examined (Bansal and Gupta, 2000; Shafique et al., 2005; William, 2008; Abd El-Ghany et al., 2015). In literature, the mycelial growth of various species of *Fusarium* was inhibited by various plant extracts such as those from *Allium sativum* and *Sapindus trifoliata* (Gohil and Vala, 1996), neem seed (Gour and Sharmaik, 1998), *Eucalyptus amygdalina* (Bansal and Gupta, 2000) or *Azadirachta indica* and *Jatropha curcas* (Abd El-Ghany et al., 2015). The inhibition of the mycelial growth may be attributed to the presence of allelochemicals with detrimental effects on cell division, cell elongation and nutrient uptake (Blake, 1985). The variation in antifungal activity of the extracts in different solvents may be attributed to the chemical nature of the three solvents (Jabeen and Javaid, 2008). Moreover, results reveal that antifungal activity of the crude extracts was enhanced by increasing the concentration of the extracts. This finding is in agreement with the report of Banso et al. (1999), and Martins et al. (2015), who also observed that the highest concentration of antimicrobial substances showed more growth inhibition. In addition, the antimicrobial activity of plant extracts might not be due only to the action of a single active compound, but also to the synergistic effect of several compounds that are in minor proportion in a plant (Davicino et al., 2007).

The considerable importance of *C. quadrangulare* in folk medicine and the paucity of reports on the phytochemical constituents of the genus prompted us to test the biologically activity of extracts. Three flavone glycosides (Shalaby and Bahgat, 2003), one iridoid and two iridoid glucosides were isolated and identified from aerial parts of *C. quadrangulare* (Ayers and Sneden, 2002). Seven iridoid glucosides, were isolated from the fruits of *Citharexylum caudatum* (Ayers and Sneden 2002). From the aerial parts of *Citharexylum spinosum* L. (Syn. of *C. quadrangulare*) five iridoid glucoside, a lignan glucoside were identified (Balázs et al., 2006); these compounds may be responsible for the antifungal activity. Indeed, flavonoids have the ability to inhibit spore germination and growth of plant pathogens (Hussin et al., 2009; Kanwal et al., 2010; Bernini et al., 2015; Wang et al., 2015). Iridoids and iridoid glucosides have been proven also for their antifungal activity (Bolzani et al., 1996; Kawamura and Ohara, 2005). Gond et al. (2015) reported that the bacterial endophytes that naturally occur in many maize varieties may function to protect hosts by secreting antifungal lipopeptides that inhibit pathogens. The most pronounced antifungal properties reported for the decoction of sage (*Salvia officinalis* L.), was positively related with its highest concentration in phenolic compounds. Fungicidal and/or fungistatic effects proved to be dependent on the extracts of concentration (Martins et al., 2015).

Alkhail (2005) studied the antifungal activity of five plant extracts against *Fusarium oxysporum* f.sp. *lycopersici, Botrytis cinerea* and *Rhizotonua solani.* Results reveal that plant extracts obtained with cold distilled water had strong antifungal activity with significant inhibition on the growth of the three tested fungi. Similar antifungal properties of ethanolic leaf extracts of *Mangifera indica* against *A. niger, Alternaria alternata, Fusarium chlamydosporum, Rhizoctonia bataticola* and *Trichoderma viride* were reported by Aqil and Ahmad (2003). Shafique et al. (2005) have reported significant inhibition of seed-borne fungi of maize *viz*., *A. fumigatus*, *A. niger*, *Rhizopus arrhizus* A. Fischer and *Penicillium* sp. by aqueous extracts of *Melia azedarach.* More recently, Khan et al. (2013) reported that crude extracts and some fractionated samples of *Tamarix dioica* Roxb. leaves showed significant antifungal properties.

Conclusion

The present study demonstrates that aqueous and organic extracts of *C. quadrangulare* may possess antifungal potential and contain inhibitory substances. This result suggest that *C. quadrangulare* could be one of the useful natural resources for developing bio fungicides, besides crude extracts of this tree could be a cost effective way for crops protection against fungal pathogens. Further research in order to know the inhibitory substances from *C. quadrangulare* organs are underway.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Efficacy of *Aeromonas hydrophila* S-layer bacterins with different protein profiles as a vaccine in Nile tilapia (*Oreochromis niloticus*)

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Farming of Nile tilapia is in full development throughout the world. However, an increase is still seen in the prevalence and severity of bacterial diseases. The aim of this study was to develop a vaccine against *Aeromonas hydrophila* in Nile tilapia (*Oreochromis niloticus*) based on protein patterns of S-layer proteins. The proteins were extracted using glycine and NaOH with subsequent visualization through SDS-PAGE. Based on the protein patterns observed, bacterins were produced that were tested in an experiment *in vivo* with the use of 144 fingerlings of Nile tilapia that were distributed in 24 aquariums in a completely randomized design. Eight distinct protein patterns were observed in SDS-PAGE, with apparent molecular mass from 52 to 72 kDa. All the unvaccinated fish inoculated with *A. hydrophila* died within 24 h after inoculation. The bacterins produced reduced the probability of death of the vaccinated fish when compared to unvaccinated ones. Hepatic histological analysis showed that the use of vaccines was able to revert changes in the liver of the fish.

Key words: S-layer protein, SDS-PAGE, *Aeromonas hydrophila,* vaccine, *Oreochromis niloticus.*

INTRODUCTION

In recent years, Brazil has stood out in the area of fishery production in the world, achieving greater prominence in the international market (MPA, 2011). Among the species most commonly farmed in the country are those of exotic origin, such as Nile tilapia (*Oreochromis niloticus*, Linnaeus, 1758). A tropical climate species that has adapted very well to conditions in Brazil (IBAMA, 2007; MPA, 2011).

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With the growth of intensive fish farming, disease outbreaks particularly, bacterial one are encountered in production systems, *Aeromonas hydrophila* are considered to be the most virulent species (Pavanelli et al., 2008). The *Aeromonas* have multifactorial pathogenesis and diverse virulence factors, including S-layer proteins (Pablos et al., 2009). The presence of the S layer at the surface of bacterial cells is strongly correlated with their virulence Proteins present in this layer are involved with different biological roles related to water reserve, nutrient functions, increase of surface adherence through formation of biofilms, invasive capacity of pathogenic bacteria, and microbial resistance to antimicrobial drugs (Kinns and Howorka, 2008).

Immunoprophylaxis of fish by vaccines is an alternative approach for preventing bacterial infections (Figueiredo and Leal, 2008). There are vaccines against some pathogens available on the market; however, they have not been shown to be effective in Brazilian samples. Because of variability, a vaccine produced abroad may be ineffective in Brazil (Figueiredo and Leal, 2008).

The aim of this study was to evaluate the effect of two bacterins produced as based on the profile of S-layer proteins from isolates of *A. hydrophila* obtained from aquatic organisms in protection of challenged Nile tilapia *(O. niloticus)*.

MATERIALS AND METHODS

Samples

S-layer proteins were extracted from 20 isolates of *A. hydrophila* originating from the bacterial collection of the Microbiology and Animal Immunology Laboratory of the Universidade Federal do Vale do São Francisco. These isolates were obtained from the kidney, integument, intestine, and lesions of Nile tilapia (*O. niloticus*) and Pac-man catfish (*Lophiosilurus alexandri,* Steindachner, 1876) showing clinical signs, collected from the Sobradinho Dam area (Sobradinho, Bahia, Brazil) and from the Centro Integrado de Recursos Pesqueiros e Aquicultura (CIRPA) (Integrated Center of Fishery and Aquaculture Resources) located in the Bebedouro district (Petrolina, Pernambuco, Brazil) in 2009 and 2010. The *A. hydrophila* isolates were previously identified through their morphological, tinctorial, and biochemical characteristics, according to Quinn et al. (1994).

One hundred forty-four (144) fingerlings were used in the experiment to test the effectiveness of the vaccine. Fish were collected from the Centro Integrado de Recursos Pesqueiros e Aquicultura (CIRPA) of Petrolina , PE, Brazil.

Extraction of S-layer proteins

S-layer proteins were extracted according to the methodology described by Fujimoto et al. (1991) and McCoy et al. (1976), with small modifications. The isolates were seeded in a TSA culture medium (*Tryptic Soy Agar*) and placed in a laboratory incubator at 28ºC for 48 h. After that, the isolates were suspended in 10 mL of 0.2 M glycine (pH 2.2) until obtaining final concentration of 5 x 10^9 CFU mL^{-1}. Soon after, the samples were placed in a vortex (20 min) at ambient temperature, and the cells were collected through centrifugation (5000 x *g* for 30 min). The pH of the supernatant was adjusted to 7.5 with NaOH (4 N), and the proteins were precipitated (overnight at 4°C) through addition of $(NH_4)_2 SO_4$ (5 g $10mL^{-1}$). After centrifugation (5000 x *g* for 1 h), the proteins precipitated were resuspended in 500 µL of 50 mM Tris-HCl pH 7.5 and stored at -20°C. The protein concentration was determined by the Bradford method (Bradford, 1976) using bovine albumin (1mg mL^{-1}) as a standard.

The proteins were visualized in 12% polyacrylamide gel with denaturing conditions (SDS-PAGE) according to the methodology described by Laemmli (1970). After preparation of the gel, the protein extracts (10 µL) were added to a mix containing 480 µL of sample buffer and 20 µL of β-mercaptoethanol heated in a water bath at 100°C for 5 min before being applied in the gels. After electrophoresis, the gels were stained with Coomassie Blue (Blum et al., 1987) and the protein standards were registered in an image-capturing system.

Vaccine production

The bacterins were prepared according to Normative Instruction 31/2003 of the Ministério da Agricultura Pecuária e Abastecimento (MAPA, 2003) [Ministry of Agriculture]. First, the strains of *A. hydrophlia* were cultivated in TSA medium at 28□C for 24 h. After growth, a suspension of the colonies in sterile saline solution was prepared and the quantity of bacteria was estimated in colony forming units per milliliter (CFU mL^{-1}) comparing a dilution of the suspension with scale 4 of McFarland through the turbidimetric method and through spectrophotometry (580 nm), optical density of 1.730; corresponding to 1.2 x 10^9 CFU. Then, 10% of this bacterial suspension was added in saline solution (NaCl 0.85%) in *Tryptic Soy Broth* (TSB) medium for cultivation in a shake flask (180 rpm) at 28ºC for 8 h. Ten mL of the vaccine culture was then removed for bacterial count, preparing decimal dilutions up to 10^6 added to TSA medium at 28ºC for 24 h. At the same time, 0.6% of formol P.A. was added to the vaccine culture for inactivation and placed once more in a shake flask (180 rpm) at 28ºC overnight. An aliquot of the treated culture was seeded in BHI (*Brain Heart Infusion*) medium and thioglycolate to confirm inactivation of the bacterial cells. After confirmation of inactivation, 15% aluminum hydroxide was added to the bacterins as an adjuvant. The bacterins were then seeded in BHI culture medium and incubated at 28ºC for 24 h to check for their safety. The bacterins were subsequently kept at 4ºC, according to the methodology of Grabowski et al. (2004).

Evaluation of the effect of the vaccine on Nile tilapia inoculated with *A. hydrophila*

To test the bacterins effect on fish, an *in vivo* experiment was carried out using 144 Nile tilapia fingerlings, sexually reversed, with a mean weight of 8.925 g and distributed in 24 aquariums of 60 L useful volume in a completely randomized experimental design The treatments consisted of six groups: two of them unvaccinated (one inoculated with saline solution and other inoculated with *A. hydrophila*); two vaccinated groups (one vaccinated with the bacterin 1 and the other with the bacterin 2) and inoculated with saline solution, and other two vaccinated groups (one vaccinated with the bacterin 1 and the other with the bacterin 2) and challenged with *A. hydrophila*, in a total of six treatments and four repetitions. The aquariums had constant aeration through air stones connected to mini air compressors. Experimental management consisted of daily siphoning in the morning (8:00) and afternoon (15:00), with the removal of around 40% of the water, which in addition to exchanging the water, also removed feces and possible leftover feed. The internal tank walls were cleaned weekly to avoid the rise of periphyton. The fish were given *ad libitum* access to commercial feed for omnivorous fingerlings.

Table 1. Parameters of hepatic histology of vaccinated and unvaccinated Nile tilapia after challenge with A. hydrophila.

Group	Parameter
Fish not vaccinated and not inoculated with *A. hydrophila*	0
Fish vaccinated with bacterin 1 and inoculated with *A. hydrophila*	1 and 2
Fish vaccinated with bacterin 2 and inoculated with *A. hydrophila*	1, 2, and 3
Fish not vaccinated and inoculated with *A. hydrophila*	3 and 4

For inoculation of the bacterins, the fish were anesthetized by immersion in benzocaine (100 mg L^{-1}) and then the vaccines were injected in the peritoneal cavity, one centimeter below the pelvic fin. The fish remained without food for 24 h before the injection and they were challenged with *A. hydrophila* 15 days after immunization. The Nile tilapia were inoculated with *A. hydrophila* through a bacterial inoculum preparation with dilution in sterile saline solution at a concentration of 2.8×10^9 CFU mL^{-1}. This solution was applied through intramuscular injection, right laterodorsal, in each experimental fish, just as the pure saline solution (control), at the proportion of 0.2 mL^{-1} for animal. After this challenge, the fish were observed every 12 h over a period of five days in regard to mortality and the occurrence of clinical signs. They were also observed for the appearance of lesions and other pathological changes brought about by *A. hydrophila.*

Statistical analysis

In order to verify the effect of the vaccine on fish infected with *A. hydrophila*, the zero inflated binomial (ZIB) model was used for statistical analysis. The ZIB model is a parametric model which properly accommodates the overdispersion caused by count data with excess zeros, as the case of our experiment. A detailed discussion regarding zero inflated models can be found in Hall (2000) and references therein. Let Y_i represent the number of dead fishes observed in the i-th tank. Then, since there are 6 fishes in each tank at the begining of the experiment, according to the ZIB model, the probability of observing Y_i dead fishes is given by:

$$P(Y_i = y_i) = \begin{cases} w + (1-w)(1-\pi_i)^6, & y_i = 0 \\ (1-w)\binom{6}{y_i}\pi_i^{y_i}(1-\pi_i)^{6-y_i}, & y_i = 1, 2, \cdots, 6 \end{cases},$$

in which w corresponds to the probability of the number of dead fishes being equals to zero, regardless of the conditions of the experiment, and π_i is the probability of those fishes subject to the conditions, that is type of vaccine (placebo, vaccine 1 or vaccine 2) and bacteria inoculation (inoculated or not inoculated) dying, given by:

$$\pi_i = \frac{\exp(\beta_0 + V_{1i}\beta_1 + V_{2i}\beta_2 + I_i\beta_3)}{1 + \exp(\beta_0 + V_{1i}\beta_1 + V_{2i}\beta_2 + I_i\beta_3)},$$

Where,

$$V_{1i} = \begin{cases} 1, & \text{if the fishes in the i-th tank receive vaccine 1} \\ 0, & \text{otherwise} \end{cases},$$

$$V_{2i} = \begin{cases} 1, & \text{if the fishes in the i-th tank receive vaccine 2} \\ 0, & \text{otherwise} \end{cases},$$

and

$$I_i = \begin{cases} 1, & \text{if bacteria were inoculated in the fishes in the i-th tak} \\ 0, & \text{otherwise} \end{cases}.$$

Histological analysis

Livers collected from an animal of each group were fixed in 10% formalin and received routine histological treatment. They were included in paraffin, cut, and stained through a modification of the Masson-Goldner Trichrome method (Pierce et al., 1978). Photomicrographs were taken of the slides obtained with 40x magnification using the IS Capture® software.

A histological classification system was used to objectively estimate the histological changes or lesions identified in liver samples of the fish evaluated (Table 1). Using this classification system, adapted from Pierce et al. (1978), a numerical value was attributed to each liver according to its histological characteristics. This system clearly differentiates between normal hepatic histology and histological changes.

RESULTS

Extraction of S-layer proteins

Quantification of the S-layer proteins of the 20 isolates of *A. hydrophila* allowed observation of concentrations that ranged from 2.954 to 3.560 µg mL^{-1}.

Differences in the protein patterns among the 20 isolates of *A. hydrophila* were visualized in SDS-PAGE. In all, eight distinct protein patterns were observed, with the presence of protein bands with predominant apparent molecular weight of 52 and 70 kDa. Eight isolates representing these profiles were then chosen to make up two vaccine groups. In electrophoresis, it may be observed that profile 1 represented four isolates with proteins bands of 52 and 72 kDa; profile 2, three isolates with 72 kDa; profile 3, two isolates with bands of 48, 52, and 70 kDa; profile 4, one isolate with 48, 52, 72, and 90 kDa; profile 5, three isolates with 48, 52, and 70 kDa; profile 6, two isolates with 52 and 70 kDa; profile 7, four isolates with 52 and 70 kDa; and profile 8, one isolate with 52 and 70 kDa. The profile of SDS-PAGE electrophoresis protein patterns of the eight isolates of *A. hydrophila* is shown in Figure 1.

Evaluation of the effect of vaccine in Nile tilapia inoculated with *A. hydrophila*

In the *in vivo* experiment, the two bacterins produced

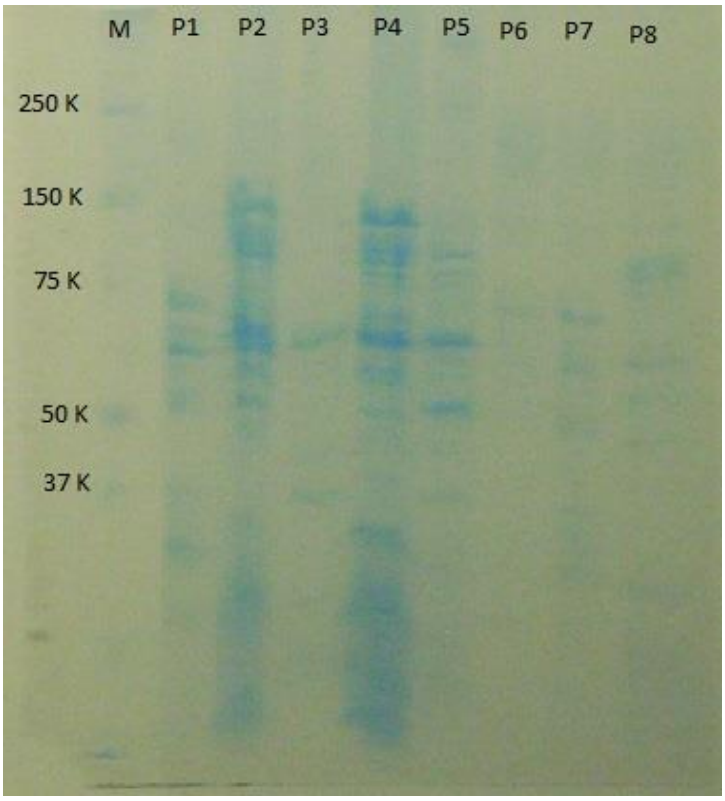

Figure 1. SDS-PAGE of eight distinct electrophoretic profiles of S-layer proteins of *A. hydrophila*: letter M refers to the molecular weight marker proteins Precision Plus Protein Dual Color Standards 161-0374 (Bio Rad) followed by profiles observed.

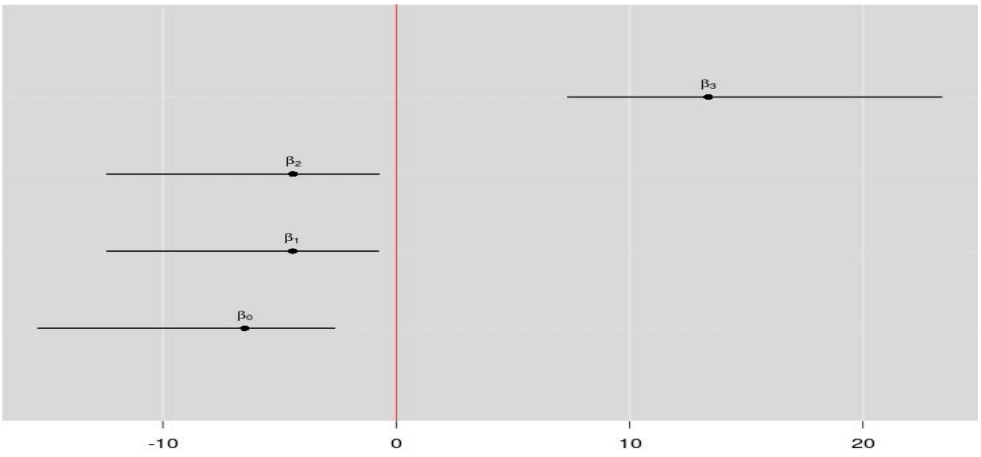

Figure 2. 95% credible interval for the regression coefficients: (β0) control group, fish not vaccinated and not inoculated with A. hydrophila, (β1) effect of vaccine 1, (β2) effect of vaccine 2, and (β3) effect of inoculation of the bacteria.

significantly reduced the probability of death of the vaccinated fish in relation to the unvaccinated ones after the challenge with *A. hydrophila*, as shown in Figure 2.

It is also observed that the probability of death of the vaccinated fish inoculated with *A. hydrophila* reduced, whereas the probability of death increased in the unvaccinated and inoculated fish. All the unvaccinated fish inoculated with *A. hydrophila* died within 24 h after inoculation. There was no statistical difference between the effect of the two vaccines in the vaccinated and

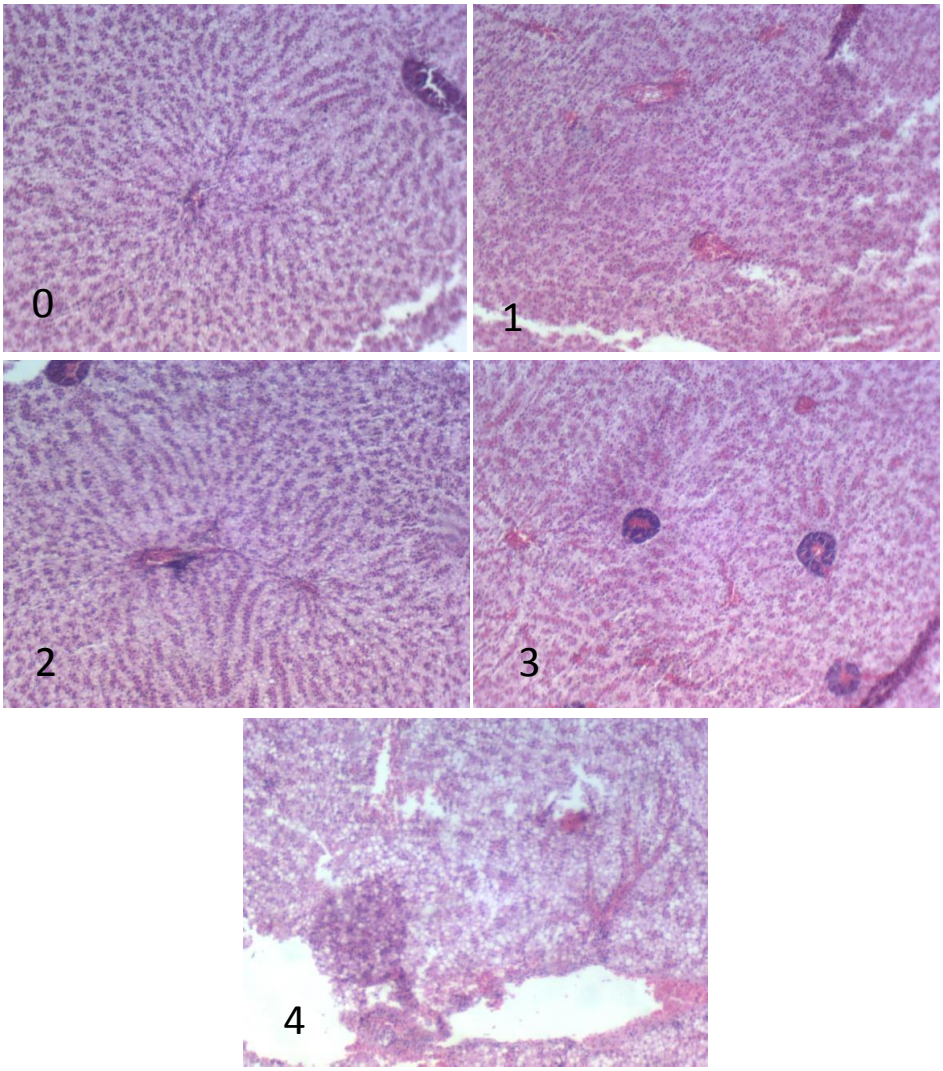

Figure 3. Hystological results of liver. Numbers: parameters described in Table 1. *Liver congestion; ** oss of hepatic cord structure.

inoculated fish. The fish that were vaccinated and that survived the inoculation showed lesions characteristic of the bacteriosis, such as redness in the ventral region, loss of scales, and hemorrhages at the base of the fins and integument, with exposure of muscle tissue in some. All the unvaccinated fish inoculated with *A. hydrophila* that died within 24 h after the challenge showed signs of systematic infection by *A. hydrophila,* such as swelling of the body, ulcerous skin lesions, and hemorrhages.

Histological analysis

Hepatic histology of the animals of the control group showed normal parameters, and these animals were classified in parameter 0, described in Table 1. The livers of the animals that were inoculated and that did not receive the bacterins showed classification 3 (25%) and 4 (75%). The major alteration was the congestion of hepatic blood vessels and loss of hepatic cord structure. It may be observed that the use of the vaccines was able to revert these changes. The inoculated animals vaccinated with bacterin 1 showed classification 1 (50%) and 2 (50%), whereas those vaccinated with bacterin 2 showed classification 1 (50%), 2 (25%), and 3 (25%) (Figure 3). These samples demonstrate a decrease in hepatic vessel congestion and increase in hepatic cord structure.

DISCUSSION

S-layer proteins are common structures in *Aeromonas* species (Esteve et al., 2004) and are correlated with the

pathogenicity of these bacteria. In this study, the concentration of the S-layer proteins extracted from 20 isolates of *A. hydrophila* showed values that ranged from 2.954 to 3.560 µg mL^{-1}, which were considered good results since such proteins represent around 10% of the total of cell proteins in bacteria (Avall-Jaaskelainen and Palva, 2005).

It is known that electrophoresis undertaken in polyacrylamide gel is a refined technique that allows determination of protein profiles of bacterial cells or extracellular products of important pathogens such as *Aeromonas* and *Escherichia coli* (Figueiredo and Leal, 2008). In this study, eight distinct electrophoretic profiles were visualized in the 20 isolates of *A. hydrophila* analyzed with protein bands with apparent molecular weight of 52 and 70 kDa. According to Ewing et al. (1960), through these profiles, the difference of antigenic structures or total proteins of isolated groups or of species may be compared.

Dooley and Trust (1988) reported that highly virulent isolates of the species *A. hydrophila* are capable of producing S-layer proteins with apparent molecular weight of 52 kDa. Other reports in the literature show that S-layer proteins in *A. hydrophila* may show variation in the molecular weights described, which are approximately 51.5 kDa (Yan et al., 1996) and 91 kDa (Rahman, 1998), depending on the isolate. Our findings indicate a variation in the molecular weight of the S-layer proteins of *A. hydrophila*, although they show predominantly a protein with apparent molecular weight of approximately 52 kDa. Data from the literature show that the S-layer proteins responsible for the main virulence factors in *A. hydrophila* are found around this apparent molecular weight (Poobalane et al., 2008; Yeh and Klesius, 2011).

According to Fagan and Fairweather (2014), bacteria that exhibit the S layer are more virulent since the presence of these proteins are implicated in different biological roles, whose functions are to act as a water and nutrient reserve, increase adherence to the surface through formation of biofilms and of force of infection, increase the invasive capacity of the pathogenic bacteria that easily escape the action of the phagocytes, and increase microbial resistance to antimicrobial agents (Kinns and Howorka, 2008; Fagan and Fairweather, 2014). The loss of ability to produce the S layer reduces the virulence of *Aeromonas* spp. (Merino et al., 1995; Noonan and Trust, 1997).

The presence of these proteins with different protein profiles in association with the antigenic variation in *A. hydrophila* isolates is still little reported in the literature. Kostrzynska et al. (1992) found antigenic diversity upon analyzing the S-layer proteins of pathogenic isolates of *A. hydrophila* and of *A. veronii* biotype *sobria,* revealing the antigenic complexity of these proteins present in these isolates. One of the possible explanations for this diversity is the great variation in gene expression among the different isolates, which, for its part, leads to different levels of expression of the virulence factors, such as surface proteins, especially the S-layer proteins (Chu et al., 1993; Boot and Pouwels, 1996; Engelhardt, 2007). As the S-layer proteins in the species of *Aeromonas* are characterized through being a common antigen found in all the isolates (Poobalane et al., 2010), interest grows in utilizing this antigen for vaccine production.

Studies related to fish immunology are in full development; nevertheless, it is known that the use of vaccines against bacterial and viral diseases, though of recent origin, has had good results in relation to scientific and economic aspects upon minimizing the use of antimicrobial drugs in the growth environment, especially due to the projections made in regard to growth of Brazilian and world aquaculture. Immunization programs against diseases and products for preventive use that are ecologically safe for maintaining the health of aquatic animals will be necessary (Aoki et al., 2008; Figueiredo and Leal, 2008; Ismail et al., 2010; Silva et al., 2013). With a view toward their clinical and economic importance, different types of vaccines have been developed against *A. hydrophila* for use in fish (Poobalane et al., 2010; Fernandez et al., 2014). Although these different vaccines show varied degrees of protection in fish, there is still not a commercial vaccine available for *A. hydrophila* (Fang et al., 2004). This is related to the inability of the vaccines in offering cross protection against different isolates of *A. hydrophila* since this bacteria is very heterogeneous in nature, both biochemically and serologically (Poobalane et al., 2010; Fernandez et al., 2014).

The bacterins produced based on the electrophoretic profiles of the *A. hydrophila* isolates in this study reduced the probability of death of the vaccinated fish compared to the unvaccinated fish. It should be noted that the challenge offered through *A. hydrophila* in the Nile tilapia fingerlings that were vaccinated and inoculated was made at a high concentration of the bacterial inoculum, with $2.8x10^9$ CFU/mL, which is considered a lethal dose. The fish that were not vaccinated but only inoculated were dead 24 h after the challenge, which confirms the large potential of pathogenicity of *A. hydrophila* derived from fish and shows its high capacity for causing diseases in these animals, especially because these isolates were obtained from sick fish. In the fish that survived that were vaccinated and inoculated, it was possible to observe lesions 24 hours after the challenge. These lesions showed characteristics common to those caused by *A. hydrophila,* and as of the third day, constancy was observed in the number and in the appearance of the lesions, lasting up to the fifth day. These findings are similar to those described for septicemic infections brought about by *Aeromonas* spp. (Boijink et al., 2001, Boijink and Brandão, 2004).

Although the vaccine was produced from the bacterial culture in broth and inactivated with formalin, it provided

protective capacity to the Nile tilapia fingerlings against the highly virulent isolate of *A. hydrophila*, which corroborates the results of Dehghani et al. (2012), who evaluated the effectiveness of vaccines inactivated by formalin and by heat, and vaccines constituted by lipopolysaccharides against *Aeromonas hydrophila*. Prasad and Areechon (2010), upon analyzing the effectiveness of a vaccine inactivated with formalin against *A. hydrophila* and *Streptococcus* sp. in red tilapia, obtained good results in stimulation of specific humoral immunity.

Although it is not possible to observe a statistical difference between the effects of the two bacterins, they confer protection to the vaccinated fish regardless of the surface protein profile. Studies indicate that the S-layer proteins together with the other surface proteins are important for protection against infection by *A. hydrophila* since these proteins are very important for initial colonization of the bacteria (Noonan and Trust, 1997). In the same way, Poobalane et al. (2010), in Japan, obtained success in effectiveness of the recombinant vaccine with S-layer protein of 45 kDa purified against different virulent isolates of *A. hydrophila* in common carp, emphasizing the importance of the use of this antigen in production of vaccines for protection against this disease. This information is extremely important for future studies with a view toward purifying and using these proteins as a common antigen, seeking satisfactory results in the immunological response of the fish when exposed to diverse species of *Aeromonas* spp., including *A. hydrophila.* The protein characterization of the isolates of *A. hydrophila* of the region will allow the proposal of a possible vaccine against these agents in fish, which may be produced efficiently, and considered alternative measures to be used by fish producers in their farming systems.

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Full Length Research Paper

Occurrence of *Salmonella* sp. and *Escherichia coli* in free-living and captive wild birds from 2010-2013 in Guarapuava, Paraná, Brazil

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Birds are increasingly close to men, and many times are raised as pets. However, many times, these animals may carry and spread enterobacteria that are deleterious to human health. *Salmonella* sp. is considered one of the most common zoonoses in the world, causing important losses to public health. Although, *Escherichia coli* is an important commensal in the gastrointestinal tract of most animals, it may cause disease both in men and animals, depending on the strain and its pathogenicity. Therefore, the objective of this study was to assess the occurrence of *Salmonella* sp. and *Escherichia coli* in free-living and captive wild birds in the city of Guarapuava, PR, Brazil. Animals were divided according to the taxonomic order, as follows: Columbiformes (228), Psittaciformes (128), Passeriformes (63), Piciformes (26), Falconiformes (19), Stringiformes (6), and Accipitriformes (01), in a total of 471 birds. Bacterial isolation was carried out by means of cloacal swabs, with 69.38% birds positive for *E. coli* and 22.32% for *Salmonella* sp. From the total of birds, 143 showed co-infection with *Salmonella* sp. and *E. coli.* Columbiformes showed the greatest occurrence of *E. coli* (82.33%). Falconiformes showed the greatest number of negative birds (57.9%). These results demonstrate that birds that were analyzed may carry and spread these enterobacteria, and preventive measures for human exposure should be determined, as these microorganisms are public health concerns.

Key words: *Escherichia coli*, *Salmonella* sp., zoonosis.

INTRODUCTION

There are 1901 birds species in Brazil, according to the latest data of the CBRO - Brazilian Ornithological Records Committee (Bencke et al., 2010). According to the Abinpet (Brazilian Association of Pet Products Industry), there were 19.1 million possible bird pets, such as cockatiels, parrots, canaries and macaws, in 2012.

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Due to the close contact between these animals and men, the occurrence of zoonoses may be favored.

According to Sick (2001), pigeons are synanthropic birds that are found in large urban areas in Brazil. These birds make their nests in cliffs, and this is the probable reason for their adaptation to urban life, as there are high buildings. Besides, other factors, such as feeding, abundance of shelter and absence of predators enable their disorderly development and reproduction. Therefore, given the close contact between these animals and men, zoonoses may occur in high rates. The same interpretation is valid, in Brazil, for Passeriformes.

Similar to all other vertebrates, birds are susceptible to and may transmit to humans enteropathogenic organisms of zoonotic potential, but there are few comprehensive studies on the issue with wild and domestic birds (Vasconcelos, 2013). Disease studies on the human population worldwide carried out by Jones et al. (2008) showed that emerging infectious diseases are, in most cases, zoonoses (60.3%). From this total, 71.3% are transmitted by wild animals, and 54.3% of these diseases are caused by bacteria and rickettsia, reflecting an increasing number of microorganisms that are resistant to the pharmaceutical drugs available in the market. According to the European Centre for Disease Prevention and Control (2010), salmonellosis and campylobacteriosis are the two most common zoonoses, and were responsible for, respectively, 99,020 and 212,064 cases of human disease in the European Union in 2010 (ECDC, 2012).

Bacteria in the genus *Salmonella* sp., Enterobacteriaceae family, are Gram-negative, facultatively anaerobic, non-sporulating rods (Carvalho, 2006). Nowadays, the genus is divided into two species, *Salmonella enterica* and *Salmonella bongori.* The species *S. enterica*, which is the pathogenic *Salmonella* species, is divided into six subspecies (*enterica, salamae, arizonae, diarizonae, houtenae,* and *indica*), each with several serovars or serotypes. More than 2,500 serotypes are known. Most of them (about 1,500) belong to the subspecies *enterica* and are associated with clinical pictures, both in humans and in animals. This classification in serotypes is based on cell surface structures, such as antigens, flagella lipopolysaccharides, and proteins (Herrera and Jabib, 2015).

Most of these bacterial infections are caused by water or food contaminated with feces of infected animals. Similarly, contamination may also be caused by direct contact between susceptible and infected animals (Kuroki et al., 2013). Therefore, birds have an important role in the dissemination of these diseases, although many of them do not show any clinical signs. Birds may also be potential carriers of many other bacteria, besides virus and parasites (Dovc et al., 2004). According to Berchieri et al. (2001) the length of fecal shedding and the level of tissue invasion (pathogenicity) depends on the age of the bird at the moment of infection. Therefore, a bird may infect other animals or human for long periods.

Studies carried out by Sousa et al. (2010) in Jaboticabal, with 126 free-living pigeons (*Columba livia*) in urban environments, showed the isolation of *Salmonella* sp. in 10 animals (7.94%).On the other hand, Silva et al. (2014) analyzed the presence of enterobacteria in domestic birds (*Cairina moschata*) from households in cities of Ceará and did not observe the presence of *Salmonella* sp.

Escherichia coli is considered to be a commensal of the gastrointestinal tract of warm-blooded animals. However, it may also be a pathogenic agent, as it adapts to diverse conditions, which is mainly related to the loss or gain of bacterial genes. Several different *E. coli* strains cause intestinal and extra-intestinal diseases by means of virulence factors that affect a wide variety of cell processes (Kaper et al., 2004). According to Croxen and Finlay (2010), there was an impressive worldwide increase in the number of cases of these diseases, with hundreds of millions of people affected annually. Similar to humans, *E. coli* may also cause diseases in the animals. Colibacillosis is one of the main causes of mortality in birds, and is responsible for significant economic losses all over the world (Schouler et al., 2012). In studies carried out by Trampel et al. (2007) in United States, *E. coli* was isolated from 14 of 15 clinically healthy birds, confirming that these animals carry the bacterium and do not show clinical signs, which may aid disease spreading.

In Brazil, there are few studies on the presence of this bacterium in wild birds. Mattes et al. (2005) evaluated the influence of biosafety measures in the intestinal colonization of Psittacidae by *E. coli*, in the state of São Paulo. Animals studied belonged to different breeding facilities, a conservation facility and a recreational facility. Results show significant differences in the intestinal colonization by *E. coli* in different environments, with more positive birds in the recreational breeding facility. These findings demonstrate the importance of biosafety measures for these animals. Diagnosis of these enterobacteria is based on the isolation of the pathogen in cultures of feces, blood, and urine. Another method used in the detection of enterobacteria is the polymerase chain reaction, PCR (Herrera and Jabib, 2015).

In Brazil, there are screening and research centers that provide veterinary attention to animals that were captured by official organs. These animals were either apprehended or voluntarily handed in to official organs. Many of these animals are highly debilitated and need intensive care to be able to be reintroduced into nature, or even to remain in captivity. Assessment of the sanitary status of the animal, mainly in relation of the presence of pathogenic organisms of zoonotic potential, is an important action that should be carried out together with healthcare measures. This assessment should be done when animals arrive at the wildlife center where they will be housed, making it possible for prophylactic and/or

Table 1. Distribution of the samples according to the bird order and species.

Order	Most common species	Number of animals (%)
Columbiformes	(*Columba livia*, *Zenaida auriculata*)	228 (48.40)
Psittaciformes	(*Ara ararauna*, *Amazona aestiva*)	128 (27.18)
Passeriformes	(*Saltator similis*, *Turdus rufiventris*)	63 (13.37)
Piciformes	(*Rhamphastos dicolorus, R. toco*)	26 (5.53)
Falconiformes	(*Caracara plancus*)	19 (4.03)
Strigiformes	(*Athene cunicularia, Tyto furcata*)	6 (1.28)
Accipitriformes	*(Rupornis magnirostris)*	1 (0.21)
Total		471 (100%)

Table 2. Results (total and percentage) of the *E. coli* and *Salmonella sp.* isolation in the different bird orders.

Order	Positive for *E. coli* (%)	Positive for *Salmonella* sp. (%)	Negative (%)	Total
Columbiformes	191 (82.33)	31 (13.36)	10 (4.31)	232
Psittaciformes	105 (73.94)	14 (9.86)	23 (16.2)	142
Passeriformes	94 (52.22)	84 (46.67)	2 (1.11)	180
Piciformes	20 (71.42)	4 (14.29)	4 (14.29)	28
Falconiformes	8 (42.1)	-	11 (57.9)	19
Strigiformes	7 (58.34)	4 (33.33)	1 (8.33)	12
Accipitriforme	1(100)	-	-	1
Total	426 (69.38%)	137 (22.32)	51 (8.30)	614*

*From 471 animals analyzed, 143 were positive for both agents.

curative measures to be adopted, if the animal is infected. Given the lack of information on the occurrence of enterobacteria in wild birds, the objective of this study was to evaluate the presence of birds positive for *Salmonella sp.* and *E. coli* among animals seen in wildlife centers.

MATERIALS AND METHODS

Cloacal sterile swabs were collected from 471 wild, clinically healthy birds, as presented in Table 1. No bird showed clinical signs compatible with the presence of *Salmonella* and *E. coli.* The study was carried out between 2010-2013, and bacterial isolation for *Salmonella sp.* and *E. coli* was performed in order to assess the presence of these enterobacteria in wildlife centers located in the cities of Guarapuava and Tijucas do Sul, both in the state of Paraná. Soon after collection, samples were sent to the Laboratory of Infectious and Parasitic Diseases at Universidade Estadual do Centro Oeste – UNICENTRO, Guarapuava, Paraná. For isolation of *Salmonella* and *E. coli* was performed according to Freitas Neto et al. (2009) with modifications. After that, swabs were placed in sterile test tubes containing 6 mL of Selenite broth (Selenite Broth Base, OXOID®) pre-enrichment medium added of Novobiocin 0.4% (1/100 mL, v/v). Tubes were placed in incubators at 37°C for 24 h. After the enrichment period, the content of the tubes was plated onto Brilliant Green (Brilliant Green Agar, KASVI®) and MacConkey agar (Mac Conkey Agar, KASVI®) and incubated again at 37°C, for 24 h. Then, plates were analyzed for colony growth and changes in agar color produced by presence or absence of bacterial fermentation, compared with the positive control was *Salmonella* sp. Colonies suggestive of *Salmonella* were isolated in slants containing (LIA) Lysine Iron Agar (LIA, BIOLOG®) and Triple Sugar Iron agar (TSI, BIOLOG®), which were kept in the incubator at 37°C for 24 h. After this period, new readings were carried out to evaluate positive samples for *Salmonella* sp. and *E. coli.*

RESULTS AND DISCUSSION

The results of the study are shown in Table 2, according to the bird orders. In the present study, a total of 471 samples of seven bird orders were analyzed. From the total of samples, 69.38% were positive for *E. coli*, 22.32% positive for *Salmonella* sp, and 8.30% samples were negative. From the total of birds analyzed, 143 (34.29%) were positive both for *Salmonella* sp. and for *E. coli*. When the bird order is taken into account, Columbiformes showed the greatest occurrence of *E. coli*, with a frequency of 82.33% of the birds. *Salmonella* sp. was more commonly isolated among Passeriformes, in a total of 46.67% of the birds. Falconiformes showed the greatest number of negative birds, with 57.9% of them.

When Passeriformes were analyzed, there was a large number of birds positive for *E. coli* (52.22%). On the other hand, a study carried out by Vasconcelos (2013) in Ceará with Atlantic canaries (*Serinus canaria*), which belong to the Passeriformes order, showed *E. coli* prevalence equal to 3.62%, with 11 samples of cloacal swabs positive in 487 samples. In the same study, when bacterial

isolation was carried out with samples collected in the *post-mortem* examination, 18.97% of them were positive in a total of 19 birds analyzed. Braconaro (2012) evaluated 253 wild Passeriformes in São Paulo and found 10.7% birds positive for *E. coli*. Brittingham et al. (1988) in the analysis of the prevalence of bacteria in Passeriformes and Piciformes birds in the USA found 1% of 387 samples positive for *E. coli*. In the present study, the occurrence of *E. coli* in Psittaciformes showed a result similar to the one observed by Mattes et al. (2005), with 73.94% positive results. These authors analyzed the presence of *E. coli* in 85 samples of Psittaciformes in two breeding facilities in the state of São Paulo. In the first facility, 20% of the birds were positive for *E. coli*, whereas in the second one, 80% of the birds were positive, making this population show a result very similar to the findings of the present study. When Jones and Nisbet (1980) analyzed 271 birds in the London zoo, they found *E. coli* in 180 samples of healthy birds, with 66.42% positive results. Different orders were analyzed in this study, and animals that belonged to the orders Piciformes and Falconiformes were positive in 100% of the cases. In our study, positive results in these two orders were 71.42 and 42.1%, respectively.

Jones and Nisbet (1980) analyzed 26 Columbiformes and showed 81.25% animals positive for *E. coli*. These results are very close to the ones found in the present study, equal to 82.33% in 228 samples.

As for Stringiformes, seven in 12 birds were positive for *E. coli* in the present study, a total of 58.34% positive results. The bacterium was also isolated from one Accipitriforme, which was positive only for *E. coli*.

According to Croxen et al. (2013), *E. coli* may be classified into several serotypes, according to the antigens it presents. There are 173 O antigens, 80 K antigens, and 56 H antigens, yielding countless O:K:H serotypes. However, the number of pathogenic serotypes is limited, with wider occurrence of non-pathogenic strains. There are two main groups of these serotypes: those that cause diarrhea, and those that cause extra-intestinal disease (Orskov and Orskov, 1992). Therefore, even in a bird population with high number of animals positive for *E. coli*, there may be no sick animals, as many serotypes are commensals. However, the possible pathogenicity of these strains to humans cannot be ruled out.

In our evaluation for the presence of *Salmonella* sp. in Passeriformes, we detected a total of 84 (46.67%) positive samples. Brittingham et al. (1988), analyzing the prevalence of enterobacteria in Passeriformes and Piciformes in the USA, did not find the presence of *Salmonella* sp. in these birds. Almeida et al. (2015), in a study with 52 samples of Passeriformes and Psittaciformes in the city of Umuarama in Paraná, did not find birds positive for *Salmonella* sp. Different from the results found in the present study, these authors showed 73.94% Psittaciformes and 52.22% Passeriformes positive for *Salmonella* sp. It should be emphasized that, in the present study, a much larger population of birds was analyzed, with 128 Psittaciformes and 63 Passeriformes.

Padilla et al. (2004) studied some bacteria and parasites of free-living pigeons in the Galapagos and did not find any sample positive for *Salmonella*, different from the samples of the present study, which showed 13.36% *Salmonella* sp. in Columbiformes.

Albuquerque et al. (2013) analyzed the experimental infection caused by *Salmonella* enteritidis in chickens and pigeons and observed that birds shed the bacterium in the feces up to 14 days after the experimental infection, demonstrating that contamination of other birds and animals may take place and cause economic losses, besides posing an important public health risk.

Birds that were analyzed in this study did not show clinical signs of the diseases, similar to the description by Vaz et al. (2015). Many times, affected birds do no show clinical signs, but shed the bacteria in eggs or feces. Bird feces contaminate the environment and, in the case of *Salmonella* sp, may remain there for a long period, depending on the environmental conditions. That is why cleaning procedures, all-in all-out, and rodent control are essential, as these animals may also spread the bacterium.

Most *Salmonella* sp. serotypes are pathogenic to humans, and clinical signs of the disease vary according to the serotype. The serotypes *S.* Agona, *S*. Hadar and *S*. Typhimurium were considered the most important causes of foodborne diseases in humans. However, nowadays, *S*. Enteritidis is considered the predominant causing agent of this kind of disease, in several countries. There is great concern, today, about the emergence of serotypes in the genus *Salmonella* that are multiresistant to available antibiotics (Shinohara et al., 2008).

In Brazil, contact between humans and birds is frequented, mainly with Columbiformes, Passeriformes, and Psittaciformes. This fact is easily proven by the number of samples collected from birds of these orders (Table 1). Greater contact with men is due to the fact that these birds may be kept as pets (Passeriformes and Psittaciformes) or their abundant presence in public places, such as parks (Columbiformes and Passeriformes). Therefore, sanitary monitoring measures for these birds are essential, as birds in these orders were those that showed the greatest absolute number of positive samples. After the sanitary monitoring, prophylactic measures may be adopted in order to reduce the dissemination of these pathogens to the human population.

Conclusion

The enterobacteria *Salmonella* sp. and *E. coli* were isolated from feces samples of free-living and captive wild

birds, demonstrating that these animals may carry and spread these pathogens. Given their close contact with men, they may transmit the diseases to humans and other animals. Therefore, the typification of the bacteria found in this study is of great importance for the level of pathogenicity of these agents to be assessed.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Antifungal and enzyme activity of endophytic fungi isolated from *Ocimum sanctum* and *Aloe vera*

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The present study was carried out to isolate endophytic fungi from *Ocimum sanctum* and *Aloe vera* and to assess their antifungal activity against *Rhizoctonia solani*, *Fusarium oxysporum*, *Colletotrichum falcatum* and *Helminthosporium maydis.* The isolated fungi were further evaluated for production of extracellular enzymes such as amylase, cellulase, chitinase, pectinase, laccase, lipase and urease. During the study 18 endophytic fungi were isolated from leaves, stem and roots of *O. sanctum* and *Aloe vera* which are different from each other morphologically. AVR1 and AVR3 show antagonistic activity towards the *Fusarium oxysporum*. T2S1 and AVL1 were found positive for amylase, TL1, AVR1, and AV2L1 were found positive in cellulase production while only AVR3 was found positive for chitinase production. AVR3, TL2, TL3, TS1, AV2R1, T2S1 and T2L1 were positive in pectinase production, none of the isolates were found positive in laccase production, only AVL1 was found positive in lipase production and TL2, T2R1, AVL1, AVL4, T2L1 and TL3 were positive in urease production. The results of the study suggest that endophytic fungi associated with *O. sanctum* and *Aloe vera* are potential agents for antimicrobial activity and a vast source of enzyme.

Keywords: Endophytic fungi, antifungal activity, extracellular enzymes.

INTRODUCTION

Endophytes are ecological group of fungi that colonize living, internal tissue of plants without any discernible features of their presence (Gehlot and Soytong, 2008; Hyde et al., 2008). They are ubiquitous, share symbiotic relationships with their hosts (Tejesvi et al., 2005) and are found in all plant species (Naik et al., 2008; Stone et al., 2000). Infected plants benefited by exhibiting increased resistance to herbivore grazing through the production of various phytochemicals (Naik et al., 2008; Owen and Hundley, 2004). Endophytes are known to produce metabolites such as alkaloids, terpenoids, steroids, quinones, isocoumarin derivatives, flavanoids, phenols, phenolic acids, and peptides (Zhang et al., 2006). *Ocimum sanctum* and *Aloe vera* can serve as good model plants for studying the effects of fungal endophytes colonization on secondary metabolism. They have significant economic importance, a well-documented chemical profile, and some of its therapeutic chemicals are known to be affected by endophytic fungi colonization (Bauer and Wagner, 1991; Lata et al., 2003;

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Araim et al., 2009). Endophytes are symbiotic microorganisms of living plants and potential sources of biologically active natural products which are useful in medical, agricultural and industrial application. The main industries that used microbial enzymes are the food, textile, leather, pharmaceutical, cosmetics, fine chemicals, energy, biomaterials, paper, cellulose and detergent industries. The present study was carried out to evaluate the antagonistic activity of endophytes and to find the new sources of valuable extracellular enzymes from endophytic fungi to understand their functional role in the host.

MATERIALS AND METHODS

Collection of plant samples

Healthy plants of *O. sanctum* and *Aloe vera* were collected from two different places of Govind Ballabh Pant University Of Agriculture And Technology Pantnagar India that is medicinal plant research and development centre (MRDC) and garden section in sterile zip lock polythene bags.

Surface-sterilization and isolation of endophytic fungi

Isolation of endophytic fungi from *O. sanctum* and aloe vera was carried out using the protocol of Sunitha et al. (2013). Different parts of the medicinal plants such as leaves, stem and roots were cut into small pieces of 1 cm and washed under running tap water for 10 min, and sterilized in series with 70% ethanol for 1 min, 1.0 % sodium hypochlorite (NaOCl) for 1 min and further cleaned by passing through two sets of sterile distilled water. The sterile samples were placed on plate containing potato dextrose agar (PDA) media with 50 mg/l concentration of ampicillin to suppress the bacterial contamination. The parafilm wrapped Petri dishes were incubated at 25± 2 °C till the fungal mycelia starts growing from the samples.

Identification of fungal isolates

The sample of fungal isolates were mounted on the sterile slides then it was stained with lactophenol cotton blue and examined in 40X light microscopy. The fungal cultures were identified on the basis of microscopic characters, for spore shape and phenotypic characteristics, for spore type, growth colour, growth rate using standard manual (Barnett and Hunter, 1998).

Antibiosis activity

Antifungal activity was screened using dual culture method described by Fatma et al. (2010) in which both endophytic fungi and test fungi were inoculated in single Potato Dextrose Agar media. Antifungal activity was checked against four plant pathogens, *Rhizoctonia solani* which mainly causes sheath blight of rice, *Fusarium oxysporum* which mainly causes panama disease of banana, *Colletotrichum falcatum* which mainly causes red rot of sugarcane and *Helminthosporium maydis* mainly causing leaf blight in maize. Test fungi were inoculated at one side of potato dextrose agar plate and endophytic fungi was inoculated at other side of the plate and incubated for five days at 27°C. Antifungal activity was indicative as mycelia growth of test fungus in the direction of active endophyte.

Extracellular enzyme production

Amylolytic activity

Amylase activity was assessed according to method described by Sunitha *et al., (*2013**),** by growing the fungi on Glucose Yeast Extract Peptone Agar (GYP) medium (glucose-1g, yeast extract -0.1g, peptone- 0.5g, agar -16g, distilled water-1L) with 0.2% soluble starch pH 6.0. After incubation, the plates were flooded with 1% iodine in 2% potassium iodide.

Cellulase activity

Glucose Yeast Extract Peptone Agar medium containing 0.5% Carboxy-methyl cellulose was used. After 3 days of fungal colony growth, the plates were flooded with 0.2% aqueous Congo red solution and destained with 1 M NaCl for 15 min. Appearance of yellow areas around the fungal colony in an otherwise red medium indicated cellulase activity.

Chitinolytic activity

To check the chitinolytic activity, fungi were grown in colloidal chitin prepared by the method of Rodriguez-Kabana et al. (1984), by partial hydrolysis with 10 N HCl for 1.5 h at room temperature. The colloidal chitin was then washed several times with large volumes of tap water and then washed with distilled water for 5 to 7 times to adjust the pH. Chitin agar media was prepared (yeast extract, 1.5 g; chiti, 2.0 g; agar, 20 g; distilled water, 1 L). Plates were inoculated with test cultures and then incubated at 26°C upto 72 h. Appearance of clear zone surrounding the culture showed chitinase activity.

Pectinolytic activity

Pectinolytic activity was determined by growing the fungi in Pectin Agar medium (Pectin -5g, yeast extract-1g, agar- 15g, pH 5.0 in 1L distilled water). After the incubation period, the plates were flooded with 1% aqueous solution of hexadecyl trimethylammonoium and a clear zone was formed around the fungal colony indicated pectinolytic activity.

Laccase activity

To assess the laccase activity Glucose Yeast Extract Peptone Agar medium with 0.05 g 1-napthol/ L, pH 6.0 was used. As the fungus grows the colourless medium turns blue due to oxidation of 1-napthol by laccase.

Lipolytic activity

For lipase activity, the fungi were grown on peptone agar medium (peptone, 10 g; NaCl, 5 g; $CaCl_2$ $2H_2O$, 0.1 g; agar, 16 g, distilled water, 1 L; pH 6.0) supplemented with Tween 20 separately sterilized and added 1% to the medium. At the end of the incubation period, a visible precipitate around the colony due to the formation of calcium salts of the lauric acid liberated by the enzyme indicated positive lipase activity.

Urease production

The ability of the isolates to attack nitrogen and carbon bonds in amide compounds was determined using Christensen's urea broth

Table 1. Fungal isolates from different parts of plants.

Region	Site of selection			
	MRDC		**Garden section**	
Pantnagar	TULSI	ALOE	TULSI	ALOE
	LEAF TLI, TL2,TL3	LEAF AVLI,AVL2, AVL3, AVL4	LEAF T2L1	LEAF AV2L1
	STEM TSI, TS2	STEM	STEM T2S1	STEM
	ROOT TRI	ROOT AVR1, AVR2, AVR3	ROOT T2RO1	ROOT AV2RO1

Table 2. Morphological characteristics' of fungal isolates.

Isolate I.D.	Colour of growth surface on PDA plates	Spore shape /hyphae structure	Nature of growth
AVL1	White	Mycelium at 90^o	Fluffy
AVL2	White	Swellon hyphae	Fluffy
AVL3	White	Swellon hhyphae	Fluffy
AVL4	White	Septate cylindrical spore	Dry
AVR1	Black	Rounded	Powdery
AVR2	White	Rounded to eliptical	Velvety
AVR3	Black/orange	Rounded	Velvety
TL1	Black	Rounded	Powdery
TL2	White	Cylindrical	Moist
TL3	Black	Boomerang shaped	Velvety
TS1	Yellow/green	Rounded	Velvety
TS2	white	Swellon hyphae	Velvety
TR1	White/black	Mycelium at 90^0	Fluffy
AV2R1	Brown	Rounded	velvety
T2S1	White	Mycelium at 90^o	fluffy
T2R1	Pink	Canoe shaped	fluffy
T2L1	Creamish	Canoe shaped	velvety
AV2L1	Black	Rounded	powdery

(peptone, 1 g; Nacl, 5.0 g, KH_2PO_4, 2.0 g; phenol red, 2%; urea, 20% aqueous sol; pH-7.0) containing the pH indicator phenol red (Cappuccino and Sherman, 2002). Urea was separately sterilized by filter sterilisation and added aseptically to basal medium. A disc of isolated fungi were aseptically inoculated into sterile Christensen's urea broth using a cork borer and incubated for 48 h. Presence of red color shows positive for urease and yellow color as negative.

RESULTS AND DISCUSSION

A total of 18 endophytic fungi were isolated from leaves, stem and root of *O. sanctum* and *Aloe vera*, which are shown in Table 1. On the basis of different growth colour, spore type and nature of growth they are categorized using the standard manual (Table 2). Each isolate were sub cultured into a PDA agar and stored at 4°C for the further studies. Dennis and Webster (1971) describes that, fungi are known to produce a number of antibiotics, such as trichodermin, trichodermol, trichotoxin, harzianum and harzianolide. These compounds were responsible for most of the inhibition of fungal pathogen. The results of the antifungal activity showed that AVR1 and AVR3 were able to suppress mycelial growth of *F. oxysporum* (Figure 7*)* and no other fungal isolates was found active against plant pathogens *R. solani, F. oxysporum, C. falcatum* and *H. maydis*. Extracellular enzymes are synthesized inside the cell and then secreted outside the cell, where their function is to break down complex macromolecules into smaller units to be taken up by the organisms as a sole source of carbon, energy, and nutrients. Urease is a nickel-containing enzyme that catalyzes the hydrolysis of urea to yield ammonia and carbamat, the latter compound decomposes spontaneously to generate a second molecule of ammonia and carbon dioxide. The results of the urease activity (Figure 6) shows that AVL1, AVL4, TL2, TL3, T2R1 and T2L1 were found positive in urease production. Simmilar results has also been reported by Smith et al. (1993). Out of 18 endohpytic fungi only AVL3 and T2S were able to degrade starch by amylase production, which is shown by significant area of clear zone around fungal mycelia growth (Figure 1), while cellulase activity

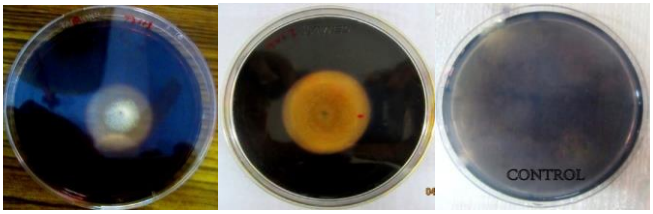

Figure 1. Amylolytic activity by endophytic fungi.

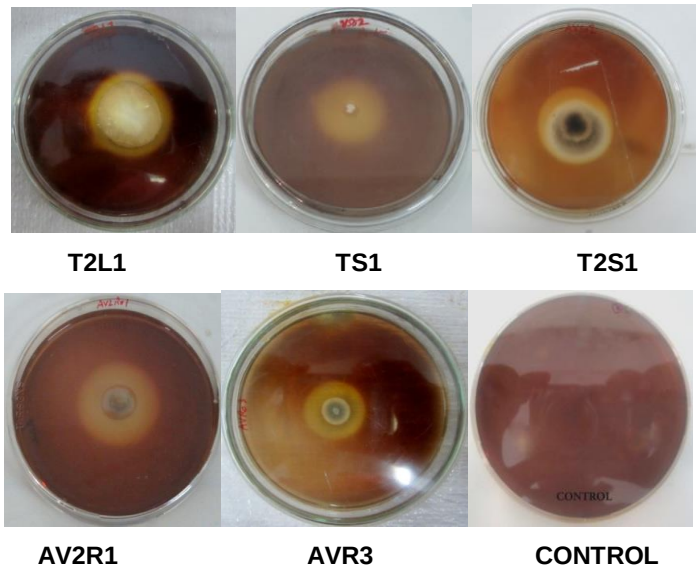

Figure 2. Pectinolytic activity by endophytic fungi.

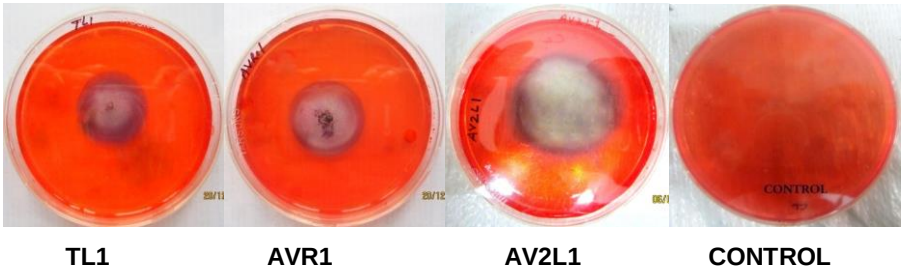

Figure 3. Cellulolytic activity by endophytic fungi.

(Figure 3) were found in four endophytes, in which AVR3 had maximum cellulase activity followed by AVR1, TL1 and TL3, which is shown by significant area of clear zone around fungal mycelia growth, Maria et al. (2006) describes the cellulose production in mangrove. Microbial pectinases are important in the decomposition of dead plant material, degradation of host tissue by phytopathogens generally begins with the production of pectinolytic enzymes. In pectinase production, maximum pectinase activity was observed in AV2R1 followed by T2L1, AVR3, TS1, T2L1, TL3, and TL2 (Figure 2). The ability of endophytic fungi to produce cellulase and pectinase may provide a resistance mechanism to the host against pathogenic invasion, to get nutrition from the host or to be a latent pathogen.(Choi et al., 2005). Lipase activity was found positive only in AVL1 (Figure 5). Amirita et al. (2012) reported lipolytic activity of *Curvularia brachyspora C. vermiformis, Drechslera hawaiiensis, C. falcatum and Phyllosticta sp.* isolated from medicinal plants. Previous report of Maria et al.

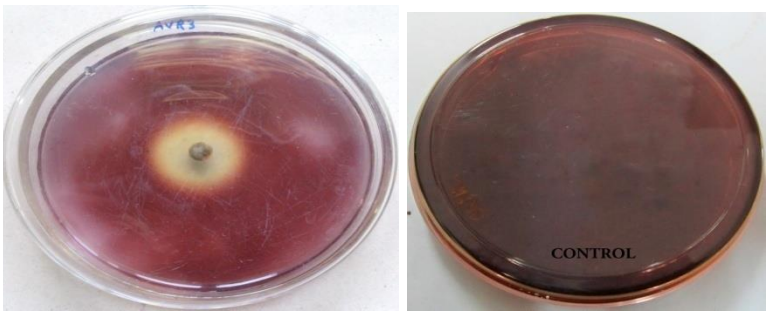

Figure 4. Chitinolytic activity by endophytic fungi.

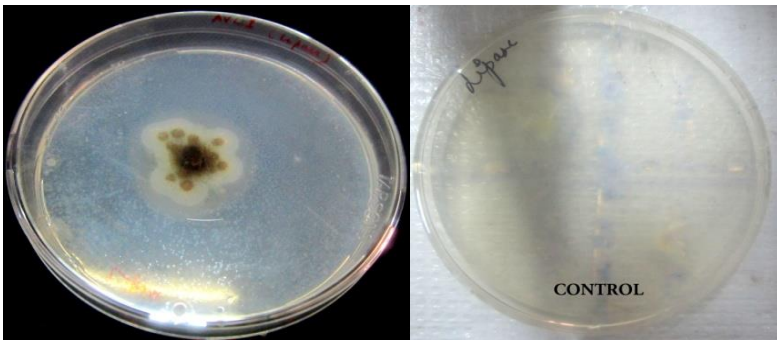

Figure 5. Lipase activity by endophytic fungi.

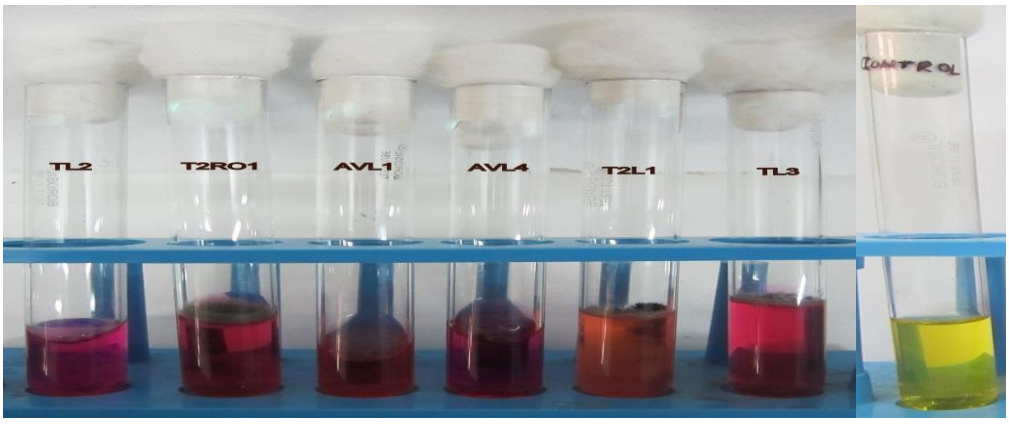

Figure 6. Urease production by endophytic fungi.

(2006), suggests that none of the isolates found active in laccase production. The endophytic nature of these fungi might be the reason for the lack of laccase activity. Out of the 18 fungal isolates only one isolate was positive for chitinase activity (Figure 4). Haran et al. (1996) stated that, chitinases play an important biological and physiological roles in fungi, containing autolytic, nutritional, morphogenetic, and parasitic roles. Chitinases in mycoparasitic fungi are most commonly suggested to be involved in mycoparasitism.

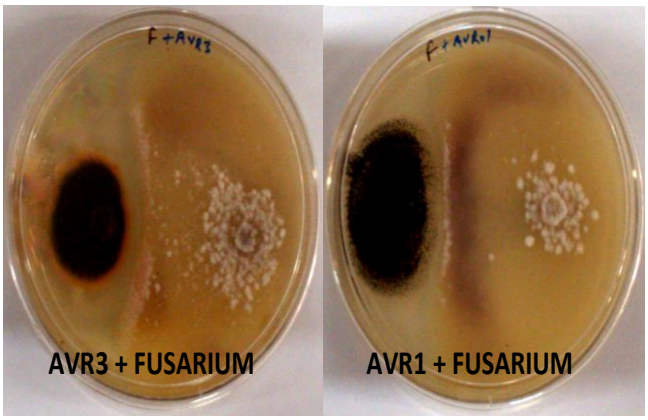

Figure 7. Antibiosis activity of endophytic fungi.

Conclusion

Endophytic fungal isolates was found to be associated with leaves, stem and root of the medicinal plant that is *O. sanctum* and *Aloe vera* and they differed significantly in their morphological, biochemical and functional characterstics. However, knowledge of the types, amounts and characteristics of enzymes produced by endophytic fungi would be useful for selecting organisms for industrial requirements. The potential endophytic fungi are being investigated quantitatively for extracellular enzyme production in liquid media.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Evaluation of the antagonistic effect of six mixed cultures of lactic acid bacteria, isolated from the Ethiopian fermented milk *ergo*, against some foodborne pathogens inoculated into the Ethiopian cottage cheese *ayib*

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The antagonistic effect of mixed lactic acid bacterial cultures against foodborne pathogens (*Staphylococcus aureus* ATCC 25923*, Shigella boydii* clinical isolate*, Pseudomonas aeruginosa* ATCC 25853) was evaluated in pasteurized *ayib* stored at room temperature. The lactic acid bacteria (LAB) were tested for acid tolerance at pH 2.0, 2.5, 3.0 and 3.5 for three and six h and for bile tolerance for 24 and 48 h at 0.3% (w/v) bile salt concentration. Their antimicrobial effect on selected foodborne pathogens was assessed by co-culturing assays in laboratory medium. The effect of mixed LAB cultures against the foodborne pathogens tested was followed in *ayib* stored at ambient conditions for 9 days. Only 11 LAB isolates were isolated upon their survival at a pH value of 3.5. Out of the 11 LAB isolates selected from a total of 60 based on their survival at pH 3.5, 6 isolates showed survival at pH 2.5 and pH 3.0 for 3 h with survival rates of 1.03-22% and 5-100%, respectively. The same 6 LAB isolates displayed tolerance to 0.3% bile salt concentration for up to 48 h. In the presence of acid-bile tolerant LAB isolates as compared to the control (without any LAB bacteria), *Ps. aeruginosa* was inhibited by all six to varying degree while, *Sh. boyidii* and *S. aureus* were inhibited by five of the LAB in laboratory medium. The mixed LAB culture was inoculated in to pasteurized *ayib* which was stored at ambient temperature for nine days and completely eliminated *Ps. aruginosa, S. aureus* and *Sh. boyidii* from day 5, 6, and 7, respectively. The result indicates that the mixed acid-bile tolerant LAB cultures eliminated the test pathogens in both laboratory medium and in *ayib*. The mixed acid-bile tolerant LAB culture could possibly be used as candidate potential protective starter culture for preparation of *ayib.*

Key words: *Ayib*, lactic acid bacteria, foodborne pathogens, acid-bile tolerance, inhibition.

INTRODUCTION

Cheese is the general name for a group of fermented milk products produced with great range of flavours,

textures, and forms. There are more than 1,000 varieties of cheese (Fox et al., 2000). Classification of cheeses as (Mexis et al., 2011). *Pseudomonas aeruginosa* has been recognized as an infectious agent transmitted by food hard, soft, semi-soft/semi-hard is purely arbitrary and utilitarian. The moisture content is the most widely accepted parameter for the categorization of cheese (Farkye and Vedamuthu, 2002). *Ayib* is a traditional Ethiopian cottage cheese made from sour milk after the removal of fat by churning, cooking butter milk and discarding the whey (Almaz et al., 2001). Traditionally, it is made from raw milk which is collected and kept at room temperature for 24 to 48 h to sour spontaneously. The pH of sour milk is usually about 4.0 (Mogessie, 2006). In traditional *ayib* making, the milk itself may have a high initial count of microorganisms and further processing may result in an increase in numbers (Mogessie, 2006). However, since cooking of the curd is expected to decrease the count of microorganisms, *ayib* is supposed to have a lower microbial load after heating (Mogessie, 2006).

Many traditional cheeses produced in local dairy plants are manufactured under poor hygienic conditions with different manufacturing technologies. These lead to potential contamination of the cheese with pathogenic microorganisms and/or their toxins, which can cause serious food borne problems to humans (Temelli et al., 2006). During the manufacturing of cheese from pasteurized milk under inadequate hygiene conditions, *Staphylocuccus aureus* may contaminate heat-treated milk or curd (Ibrahim et al., 1981). Cheese manufactured from raw milk, particularly in cases of slow or insufficient acidification of the curd, has led to staphylococcal food poisoning outbreaks associated with this product (Ibrahim et al., 1981).

Ergo is a traditional Ethiopian fermented milk product which ferments spontaneously by lactic acid bacteria (Almaz et al., 2001) and from which generally *ayib* is prepared. It is thick, smooth, white color with uniform appearance that has some similarity to yoghurt. The product is semi-solid and has a pleasant odour and sour taste (Almaz et al., 2001).

Yeasts and moulds often cause problems in a cheese product during storage (Mexis et al., 2011). Their growth on the surface of the cheese is responsible for unpleasant flavour development, changes in colour, and texture or deformation of cheese packages (Mexis et al., 2011). Furthermore, growth of psychrotrophs such as *pseudomonads* causes spoilage, showing a slimy appearance and unpleasant odour in high pH cheeses (Mexis et al., 2011). Pseudomonas aeruginosa has been recognized as an infectious agent transmitted by food and water (Wiedmann et al., 2000). This organism is an opportunistic pathogen affecting primarily immune compromised people and those suffering from cystic fibrosis (Wiedmann et al., 2000). A large outbreak of gastroenteritis caused by consumption of fresh cheese made from pasteurized milk contaminated with *Shigella* was recorded in the Murcia region of Southeast Spain (Garcia-Fulgueiras et al., 2002).

Chemical preservatives, such as sorbate and propionic acids, are occasionally used in cheese and their products to extend their shelf-life; however such additives may cause undesirable off-flavours (Mexis et al., 2011). Furthermore, consumers' growing concern over the safety of foods containing synthetic chemical preservatives, along with the economic impact of spoiled foods, have led to the investigation of alternative 'natural' cheese preservation technologies (Mexis et al., 2011).

Lactic acid bacteria (LAB) have a long history of safe use in food (Bourdichon et al., 2012), and are frequently used as bio-preservatives in food and feed storage. The general preserving ability of their fermentation end products and the antibacterial effects of LAB proteinaceous bacteriocins are well documented (Songisepp et al., 2004). Consequently, these are used as protective cultures to inactivate and reduce pathogenic bacteria, thus protecting human health (Mezaini et al., 2009).

Ayib is commonly handled and packaged in unsanitary conditions at house hold level; consequently, there is a high possibility of its being contaminated with spoilage and food borne pathogenic organisms, which reduces the shelf life of the product. Therefore, this study was aimed at finding appropriate biopreservative methods to extend the shelf-life of *ayib*.

MATERIALS AND METHODS

Sample collection

Thirteen (13) samples of *Ergo* (200 ml) were collected from 5 selected sub-cities (*Megenagna, Shola, Kebena, Arat Kilo and Saris*) of Addis Ababa using sterilized bottles. Until analyses, samples were kept under refrigeration at 4^0C in the laboratory. Twenty five (25) ml of each sample were mixed with sterile 225 ml of peptone water (0.1% W/V) to make 10^{-1} and serially diluted (Erdogrul and Erbilir, 2006). Similarly, samples of ayib were purchased from shola market, Addis Ababa, Ethiopia.

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Abbreviations: ATCC, American type culture collection; **CFU,** colony forming unit; **LAB,** lactic acid bacteria; **MRS,** de-Man Rogossa and Sharp; **MSA,** mannitol salt agar; **NaCl,** sodium chloride; **PIA,** pseudomonas isolation agar; **SS,** *Salmonella-Shigella*; **W/V,** weight by volume; **EHNRI,** Ethiopian Health and nutrition research institute; **SPSS,** statistical package for social science; **SSA,** *Salmonella-Shigella* Agar; **LSD,** list significant difference.

Isolation, counting and purification of lactic acid bacteria from *Ergo*

Isolation and counting of Lactic acid bacteria

For the isolation of LAB, 0.1 ml of appropriate dilutions (10^{-2} - 10^{-5}) of *ergo* was plated in duplicate onto the surface of pre-dried de-Man Rogassa Sharp (MRS) (OXOID) agar plates. Inoculated plates were incubated anaerobically at 32°C for 24 to 48 h using a Gas pak anaerobic jar (BBL). All colonies were counted as LAB (Girum et al., 2005a).

Purification of LAB

After colony counting, 10-20 colonies were randomly picked from countable MRS plates for further identification. Colonies of LAB were transferred into 5 ml of MRS broth (Oxoid) and were purified by repeated streaking on MRS agar. Pure cultures of LAB were then streaked onto slants of MRS agar, and stored at 4^0C for further characterization.

Morphological, physiological and biochemical examinations

The isolates were identified according to their morphology (cell shape, cell arrangements, and motility), cultural characteristics (colony size, colony color, colony texture), physiological and biochemical characteristics (KOH-test, catalase test, cytochrome-oxidase test, growth at 20 and 45°C, production of acid and gas from 1% glucose [MRS broth without beef extract] and growth in the presence of 4 and 6.5% NaCl) based on Bergey's Manual (Nair and Surendran, 2005).

***In vitro* analysis of probiotics properties of LAB**

The common methods for *in vitro* analysis of probiotic properties include acid and bile tolerance and antagonism against some test foodborne organisms were done. In this study, acid and bile tolerance and antagonism of LAB against test pathogens were done (Hyronimus et al., 2000).

Acid tolerance

All the LAB isolates were grown in MRS broth at 37°C overnight. A volume of 1 ml of log 7 cfu/ml of each overnight grown culture was inoculated into 10 ml of MRS broth to give initial inoculum level of log 6 cfu/ml in duplicate tubes acidified to different pH values (2.0, 2.5, 3.0 and 3.5) using 3.0 M HCl. Inoculation of the LAB isolates to a pH 3.5 was considered as preliminary test for acid tolerance. Inoculated tubes were incubated for 3 to 6 h at 37°C. Cells were serially diluted 10-fold in phosphate buffer (0.1 M, pH=6.2) in order to neutralize the medium acidity. A volume of 0.1 ml aliquots of appropriate dilutions (10^{-1}-10^{-3}) was spread on duplicate pre-dried MRS plates for viable cell count. Plates were then incubated under anaerobic condition in anaerobic jar (BBL, Gas Pak Anaerobic systems), at 37°C for 24-48 h (Hyronimus et al., 2009).

Bile tolerance

Acid-tolerant isolates were examined for bile-tolerance; following Dunne et al. (2001). Each acid-tolerant LAB strain was grown overnight at 37^0C in MRS broth. Duplicate tubes of MRS broth (10ml) supplemented with 0.3% bile salt conc. (sigma Chemical Co. St Louis, Missouri, USA) were inoculated at an initial inoculum level of log 6 cfu/ml, and incubated at 37°C for 24 to 48 h. A volume of 0.1 ml aliquots of appropriate dilutions (10^{-1}-10^{-3}) were spread on surface of pre-dried MRS plates for counting. Then plates were incubated under anaerobic condition in an anaerobic jar (BBL, Gas Pak Anaerobic systems) at 37°C for 24-48 h.

Determination of antimicrobial activity of LAB

Test organisms

The test organisms (*S. aureus* ATCC 25923*, Sh. boydii* clinical isolate*, Ps. aeruginosa* ATCC 25853) were obtained from Ethiopian Health and Nutrition Research Institute, Addis Ababa, Ethiopia (EHNRI).

Antimicrobial effect of LAB

LAB isolates those that were considered acid and bile tolerant were inoculated into 200 ml of modified buffered MRS broth in 250 ml flask (MRS broth with 1% of glucose, 2% yeast extract and 2% of sodium betaglycerol phosphate) in duplicate to give a final inoculum level of log 6 cfu/ml (Moreno et al., 1999). This served as a control for the LAB isolates.

Similarly, each test pathogen was separately inoculated into 200 ml modified MRS broth in duplicate to give a final inoculum of log 3 cfu/ml as a control. The experimental culture consisted of inoculation of LAB culture and the test pathogen together in 200 ml of modified buffered MRS broth in duplicate to give a final inoculum level of log 6 cfu /ml and log 3 cfu/ ml for the LAB and the test pathogen, respectively. All flasks were incubated at 32°C for 48 h.

Samples (10 ml) from each co-cultured (LAB-test pathogen) and control flask were drawn at 0, 24 and 48 h. Appropriate dilutions (0.1 ml aliquot) were plated and enumerated on Mannitol Salt agar (oxoid), Salmonella Shigella agar (oxoid) and Pseudomonads isolation agar (Difco) for *S. aureus, Sh. boydii and Ps. aeruginosa*, respectively. When growth of test enteric pathogens was not detected (<log1 cfu/ml), 1 g each sample was enriched in 9 ml of nutrient broth, incubated at 37°C overnight and streaked on the appropriate medium. Plates were checked for the presence of characteristic colonies of the target pathogenic strains.

Measurement of pH

The pH of *ayib* samples was determined by blending 10 g of *ayib* sample in a stomacher with 90 ml sterilized peptone water. The pH of the homogenate was then measured using the digital pH-meter (pH-016, Ningbo Free Trade Zone, China). *Ayib* samples were prepared for test by pasteurizing at 80°C for 10 min in order to remove vegetative cells of spoilage microorganisms.

Enrichment of ayib with LAB

Culture of *S. aureus* ATCC 25923 was grown in nutrient broth overnight at 37°C. The growth suspension was serially diluted in 10 ml sterile peptone water to give ca: 3 x 10^4cfu/ml. Similarly, LAB isolates were grown overnight, at 37°C in 10 ml MRS broth (Anteneh et al., 2011).

A mixed culture of 6 LAB isolates were prepared by transferring one ml of culture broth from each pure culture into 54 ml of sterile peptone water in screw-capped bottle. The mixture represented LAB mixed culture from all groups with an approximate population of 10^7 cfu/ml. This served as a stock culture (Girum et al., 2005a).

About 200 g of *ayib* which was bought from *shola* market and

Table 1. A Survival rate (%) of LAB isolated from *Ergo* under acidic conditions after 3 and 6 h of incubation.

Isolates	Survival rate of LAB isolates in (%)			
	3 h		6 h	
	pH=2.5	pH=3.0	pH=2.5	pH=3.0
EMA2a2	22.00	100.00	-	-
EMB1a3	-	25.48	-	-
EKU1	-	5.00	-	-
EMA6	25.40	96.15	-	14.38
EMB5	9.90	41.48	-	-
EMB6	1.03	100.00	-	22.50
EMA4	-	-	-	-
EMA5a1	-	-	-	-
EArA1	-	-	-	-
EArA3	-	-	-	-
EKB3	-	-	-	-

No survival rate, EM: Ergo from Megenagna, EAr: Ergo from Aratkilo, EK: Ergo from Kebena while A1, A2a2, A3, A4, A6, B1, B1a3, B3, B5, B6, U1, shows different colony characteristics of the isolate during purification.

was heat treated (pasteurized) in water bath at 80°C of internal temperature for 10 min. The heat treated fresh *ayib* was then cooled to 4°C in refrigerator. To the 200 g heat treated and cooled fresh *ayib*, *S. aureus* ATCC 25923 was added to give final inoculum level of 10^3 cfu/g for the control. Similarly, 200 g of *ayib* was co-inoculated with mixed culture of LAB isolates and *S. aureus* ATCC 25923 to give final inoculum levels of 10^6 cfu/g and 10^3 cfu/g, respectively.

Samples were drawn and counted at zero hour, 1st, 2nd, 3rd, to 9th day. The same procedure was applied to the other test strains (*Sh. boydii* and *Ps. aeruginosa* ATCC 25853) separately. When growth of test enteric pathogens was not detected (<log1 cfu/ml), a 1 g sample was enriched in 9 ml of nutrient broth and incubated at 37°C overnight. For detection of *S. aureus, Sh. boyidii* and *Ps. aeruginosa* enriched samples were streaked on Mannitol Salt agar (MSA), Salmonella Shigella agar (SSA) and Pseudomonads isolation agar (PIA), respectively. Plates were checked for the presence of characteristic colonies of the target strain.

Statistical analyses

Mean, standard deviation and standard error of the mean were analyzed using SPSS (version 16.0, SPSS Inc, Chicago, IL, USA, 2007). Anova and least significant difference (LSD) was performed for means comparison at ($p<0.05$) using the same program.

RESULTS AND DISCUSSION

In this study, a total of 60 isolates of LAB were isolated from 13 *Ergo* (traditional Ethiopian fermented milk) samples collected from some parts of Addis Ababa. Eleven LAB isolates were selected from a total of 60 isolates based upon their survival at pH 3.5 (not indicated in the table). Out of the eleven LAB isolates tested for tolerance at different pH values and exposure time, only six isolates showed tolerance to the tests (Table 1).

All the 6 acid-bile tolerant LAB isolates were Gram positive, grew in 4% NaCl at 20, 30, 37 and 45°C; but did not grow at 6.5% NaCl (data not shown). Out of the six isolates, 3 isolates (50%) did not release carbon dioxide, whereas the other 3 isolates (50%) did (Table 2).

All 6 LAB isolates were identified as *Lactobacillus* spp based on the morphological, physiological and biochemical characteristics. They were found to be equally divided in to homofermentative and heterofermentative types (50:50) based on carbon dioxide release. The dominance of *Lactobacillus* from different fermented products was also substantiated by similar work on Borde and Shamita (>50% of LAB isolates) (Girum et al., 2005b) in Ethiopia, and on yoghurt (83%) in Khartum (Ali, 2011). Similarly, LAB isolated from raw cow milk, white cheese and rob in Sudan were shown to be dominated with *Lactobacillus* genera (≥69.23%) (Abdullah and Osman, 2010).

Acid tolerance test

Out of the eleven LAB isolates tested for tolerance to different pH values and exposure times, only six isolates showed tolerance to the tests (Table 2). Four isolates were tolerant to growth media adjusted to pH 2.5 for 3 h incubation period with various survival rates, and no isolates survived as the incubation time increased from 3 to 6 h (Table 2). EMB6, EMB5, EMA2a2 and EMA6 isolates showed tolerance to pH 2.5 for 3 h and survived at a rate of 1.03, 9.9, 22 and 25.4%, respectively (Table 2). None of the isolates survived to pH 2 for 3 and 6 h.

A similar study indicated a 24% survival percentage of *Lactobacillus* spp. isolated from *awaze* (fermented condiment), *qotchqotcha* (fermented condiment) and *tef*

Table 2. Physiological, morphological and biochemical characteristics of the isolates.

Isolates	Cultural characteristics		Glucose fermentation	Production of CO_2 from glucose	Acid production from glucose	Remarks
	Size	Texture				
EMA2a2	F	R	HrF	+	+	*Lactobacillus*
EMB1a3	M	M	HrF	+	+	*Lactobacillus*
EMA6	S	S	HF	-	+	*Lactobacillus*
EKU1	M	M	HF	-	+	*Lactobacillus*
EMB5	S	S	HrF	+	+	*Lactobacillus*
EMB6	S	M	HF	-	+	*Lactobacillus*

HF: homo fermentative, HrF: heterofermentive, +: positive test, −: negative test, F: fine, M: medium, S: small, m: mucoid, R: rough, s: smooth, EM: Ergo from Megenagna, EAr: Ergo from Aratkilo, EK: Ergo from Kebena while A2a2, A6, B1a3, B5, B6, U1, shows different colony characteristics of the isolate during purification.

Table 3. Survival rate in (%) of different LAB isolate at 0.3% (w/v) bile salt concentration.

Isolate	Bile salt tolerance in survival rate (%)	
	24 h	48 h
EMA2a2	100%	11.5%
EMB1a3	64.5%	9.7%
EMA6	76.5%	8.5%
EKU1	100%	100%
EMB5	11%	-
EMB6	96.1%	37.7%

No survival rate, EM: Ergo from Megenagna, EAr: Ergo from Aratkilo, EK: Ergo from Kebena while A2a2, A6, B1a3, B5, B6, U1, shows different colony characteristics of the isolate during purification.

dough which was exposed to pH 2.5 for 3 h (Asnake and Mogessie, 2010). Likewise other investigators reported that the survival rate of 3 LAB strains isolated from marine fish (APa4, AIa1, and ARa1) exposed to pH 2.5 for 1 h, showed 53, 41 and 37% survival, respectively (Buntin et al., 2008).

The survival rate of isolates at pH 3.0 for 3 h was found to vary among the isolates. Out of the eleven isolates 55% were found tolerant to pH 3.0 for 3 h with different survival rate (5-100%) (Table 2). Isolates EMA2a2 and EMB6 showed a survival rate of 100%; whereas EMA6, EMB5, EMB1a3, and EKU1 showed survival rates of 96.15, 41.48, 25.48 and 5%, respectively. Similarly, *Lactobacillus* spp isolated from yoghurt showed a survival rate between 72-96% at pH 3 for 3 h (Boke et al., 2010). But it is also contrary to the report that showed only 18% of the LAB isolates from a traditional Ethiopian fermented beef sausage were tolerant to pH 3.0 for 3 h with survival rate of 60-100% (Ketema et al., 2009).

However, the average survival percentage of LAB isolates from *awaze*, *qotchqotcha* and *tef dough* was found to be 48% (Asnake and Mogessie, 2010). In addition to this, moderate survival rate of LAB isolated from cattle feces was observed for four strains with 11-26% survival rate after 3 h with the highest survival rate at pH 3.0 for 3 h as 100% (Hyronimus et al., 2000). Other reports also showed that four acid tolerant strains from 200 LAB isolates had shown 80% survival after exposure to pH 3 for 3 h (Buntin et al., 2008).

Furthermore the exposure of these LAB isolates to pH 3 for 6 h reduced the potentially useful isolates to two isolates (EMA6 and EMB6) with survival rates of 14.38 and 22.5%, respectively. This showed that the survival rate of the isolates from the present work was reduced significantly after 6 h of incubation. In comparison to the result of the present study, it was reported that the survival rate was 38% for *Lactobacillus* spp isolated from *awaze*, *qotchqotcha* and *tef dough* at pH 3 for 6 h (Asnake and Mogessie, 2010).

In addition to this, moderate survival rates of LAB were observed for six strains with 0.2-15% after 6 h at pH 3 while, the highest survival rate was 55% (Hyronimus et al., 2000). Similarly, it was shown that out of 56 *Lactobacilli* isolated from *wakalim*, a traditional Ethiopian fermented beef sausage exposed to pH 3 for 6 h, 11 isolates showed 1-20% survival rate (Ketema et al., 2009).

Bile tolerance test

The six isolates showed more consistency in tolerance to bile (0.3% bile salt) compared to acid. This could be due to the damage of HCl to the organisms (lactobacilli) been more harmful than bile acid (Kheadr, 2006). The survival rate of most of these isolates for up on 24 h of incubation was between 64.5-100% (Table 3). This was similar to the bile tolerance (47.8-100%) of different *Lactobacillus* spp. isolated from conventional yogurt samples by Ashraf et al., (2009). A survival rate of more than 60% was also shown for strains of *Lactobacilli* isolated from traditional fermented food in Thaiup on 24 h of incubation (Klayraung et al., 2008).

In the present study, four isolates were shown to

Table 4. The inhibitory activity of acid and bile-tolerant LAB against *Ps. aeruginosa, Shigella boyidii* and *S. aureus* by co-culturing in laboratory medium.

Isolate	Incubation time					
	0 h		24 h		48 h	
	Log cfu/ml	pH	Log cfu/ml	pH	Log cfu/ml	pH
EMA6&Ps	3.52±0.04	6.23	0±0.00	4.74	0±0.00	4.4
EKU1&Ps	3.44±0.14	6.24	1.44±0.06	4.82	0±0.00	4.34
EMA2a2&Ps	3.23±0.21	6.20	1.34±13	4.77	0±0.00	4.35
EMB1a3 & Ps	3.54±0.04	6.26	4.47±0.18	4.83	3.31±0.32	4.41
EMB5 & Ps	3.72±0.01	6.21	2.44±.14	4.66	0±.000	4.32
EMB6 & Ps	3.39±0.12	6.24	3.52±0.09	4.67	3.79±0.10	4.35
EMA6 & Sh	3.29±0.02	6.26	0±0.00	4.77	0±0.00	4.5
EKU1 & Sh	3.19±0.16	6.25	5.53±0.30	4.87	5.63±0.22	4.41
EMA2a2 & Sh	3.49±0.18	6.21	6.54±0.09	4.72	8.58±0.05	4.31
EMB1a3 & Sh	3.46±0.09	6.25	4.65±1.34	4.82	5.47±0.05	4.42
EMB5 & Sh	3.23±0.07	6.22	2.24±0.34	4.72	0±0.00	4.4
EMB6&Sh	3.65±0.03	6.20	6.31±0.15	4.70	0±0.00	4.38
EMA6 & Staph	3.40±0.11	6.26	3.30±0.09	4.79	1.70±0.18	4.43
EKU1 & Staph	3.32±0.21	6.26	3.89±0.14	4.78	1.97±0.10	4.46
EMA2a2 & Staph	3.31±0.11	6.33	3.56±0.04	4.81	3.72±0.36	4.42
EMB1a3 & Staph	3.36±0.15	6.23	6.80±0.05	4.86	7.60±0.25	4.43
EMB5 & Staph	3.50±0.18	6.23	2.60±0.73	4.71	0.95±1.35	4.42
EMB6 & Staph	3.50±0.02	6.23	2.33±0.02	4.69	2.22±0.04	4.39
Ps. Aeruginosa, Cont	3.45±0.08	6.23	5.43±05	5.30	7.43±0.09	5.1
Shigella boyidii, Cont	3.75±0.06	6.20	6.43±0.09	4.97	8.51±0.27	4.42
Staphylococcus aureus Cont	3.88±13	6.23	7.49±08	5.00	7.59±03	4.98

Cont: Control, Sh = *Shigella boyidii*, Staph = *Staphylococcus aureus*, Ps = *Pseudomonads aeruginosa*, EM: Ergo from Megenagna, EAr: Ergo from Aratkilo, EK: Ergo from Kebena while A2a2, A6, B1a3, B5, B6, U1, shows different colony characteristics of the isolate during purification.

tolerate bile (0.3%) up to 48 h with a survival rate of 8.5-37.7%, except isolate EKU1 that showed a dramatic survival rate of 100%. But isolate EMB5 did not survive in 0.3% bile salt concentration for 48 h. Similarly, it was reported that LAB isolated from marine fish showed 20% survival up on 48 h (Buntin et al., 2008). According to Oh et al (2000), all five strains of *L. acidophilus* exhibited excellent bile (0.3%) tolerance of ≥ 50% survival rate up on 48 h incubation period. Thus, the acid-bile tolerant LAB isolates could potentially tolerate the acidic environment of the stomach. In addition, the tolerance of these LAB isolates to 0.3% bile salt is indicative of their potential survival in the small intestine (Gilliland et al., 1984). It is possible to consider isolates as potential candidates' probiotic as they have showed reasonable survival rate during *in vitro* selection criteria. So these isolates could potentially resist the hurdles in stomach and small intestine (Dunne et al., 1999).

Antimicrobial effect of LAB against some enteropathogens

After co-culture with test organisms in a laboratory medium during co-incubation for 48 h, 4 out of 6 isolates demonstrated a better inhibitory effect on the test pathogens. But the best inhibitory effect against all the three test pathogens was observed by the isolates EMA6 and EMB5. Isolate EMA6 was the only isolate that completely inhibited (reduced 3 log units) both *Ps. aeruginosa* and *Sh. boyidii* after 24 h co-incubation. Furthermore, isolates EKU1, EMA2a2 and EMB5 totally eliminated *Ps. aeruginosa* after 48 h. Isolates EMA6, EKU1 and EMB5 were reduced about 1 log unit of *S. aureus* population up on 48 h co-incubation with LAB (Table 4). The inhibition of test pathogens by LAB in the laboratory medium might be due to the production of lactic acid, and reduction pH of the medium; and due to the production of other antibacterial substances including bacteriocin (Vuyst and Leroy, 2007).

Similarly, it was observed that the growth of different pathogen was lower than the control after co-incubating each pathogen with probiotic *Lactobacillus rhamnosus* for 24 h (Pirarat et al, 2009). However, other study revealed that *Enterococcus faecium* FAIR-E 198 did not show any significant inhibitory effect against *S. aureus* ATCC 27154 during 48 h co-culturing period, in which the present work showed better inhibitory effect against *S.*

Table 5. The inhibitory effect of mixed LAB on test pathogens inoculated in to ayib during storage at ambient temp for 9 days.

Isolates	Count of test pathogens (Log cfu/g) and pH of Ayib samples													
	Day 1		Day 2		Day 3		Day 4		Day 5		Day 6		Day 7	
	Count	pH	Count	pH	Count	pH	Count	pH	Count	pH	Count	pH	Count	pH
LAB &Staph	3.56	4.60	4.63	4.56	4.72	4.46	1.63	4.42	1.39	4.39	-	4.35	-	4.33
Staph cont	3.13	4.61	5.29	4.58	5.41	4.58	6.55	4.58	8.62	4.60	5.63	4.61	3.39	4.60
LAB & Shig	3.59	4.61	4.50	4.54	3.24	4.44	2.29	4.43	2.54	4.41	2.26	4.39	-	4.34
Shig. cont	3.70	4.60	5.71	4.60	6.81	4.58	7.82	4.60	7.91	4.61	8.99	4.60	6.40	4.58
LAB & Ps	3.57	4.62	3.54	4.50	2.82	4.41	1.55	4.40	-	4.40	-	4.38	-	4.37
Ps. cont	3.61	4.62	4.17	4.60	6.83	4.58	7.17	4.59	5.45	4.60	8.37	4.62	7.24	4.62

LAB & Staph = Ayib inoculated with co-culture of *Staphylococcus aureus* with LAB, LAB & Shigella = Ayib inoculated with co-culture of *Shigella boyidii* with LAB, LAB & Ps = Ayib inoculated with co-culture of *Pseudomonas aeruginosa* with LAB, LAB=Lactic acid bacteria, Staph = *Staphylococcus aureus*, Shig = *Shigella boyidii*, Ps = *pseudomonas aeruginosa*, Cont: control, –: the microbial count is zero (totally inhibited)

aureus (Nascimento et al., 2010). In other study, 2.6 log units reduction of *S. aureus* count by probiotic bacteria was shown during co-culturing with LAB in laboratory medium (Tharmaraj and Shah, 2009). Correspondingly, when the *S. aureus* and *Lc. lactis* were co-cultured in broth medium in the ratio (*S. aureus*: *Lc. lactis*, 1/1 and 1/10), the population of *S. aureus* was reduced by 4 log and 5 log units, respectively (Charlier et al., 2009). Likewise, the co-culturing of LAB strain L22 with *S. aureus* ATCC 25923 reduced the population of *S. aureus* by 8 log units after 24 h incubation (Voravuthikunchai et al., 2006).

In comparison to the work of other investigator that reported the weak antibacterial activity against *Ps. aeroginosa* by *Lactobacillus casei* and *Lactobacillus bulgaricus* isolated from various foods, the current results showed good inhibitory effect against *Ps. aeroginosa* (Erdogrul and Erbilir, 2006). Similarly, Hutt et al., (2005) also reported that significant log units (0.6-3.2 log units) of *Sh. sonnei* ATCC 25931 was reduced up on 24 h co-incubation with *L. plantarm* 299v.

The fate of test pathogen in mixed LAB enriched *ayib*

In this study, the test pathogens were completely inhibited from *ayib* co-inoculated with mixed LAB cultures within 5-7 days. The inhibition was highest against *Ps. aeruginosa* in reducing 3 log units within 5 days, followed by *S. aureus* by reducing 3 log units within 6 days and against *Sh. boyidii* in reducing 3 log units within 7 days (Table 5). The inhibition of test pathogens by LAB in *ayib* enriched with mixed cultures of LAB can be as a result of the production of lactic acid, acetic acid and bacteriocin by LAB that display antibacterial activity (Vuyst and Leroy, 2007). This was in agreement with another report that indicated the mean count of the test pathogens decreased by 3-4 log units at day 3 and they were totally eliminated at day 6 or 7, in mixed LAB culture dipped *ayib* and kept at ambient temperature (Anteneh et al., 2011). Similarly, kefir produced using freeze dried culture of *Lactobacilli*, *Lactocccus* and *Leuconostoc* as a starter have shown the best antimicrobial effect against *S. aureus* (Ulusoy et al., 2007). Furthermore, fermentation of *borde* by mixed LAB cultures resulted in the reduction of test pathogens (*Escherichia coli*, *Salmonella typhimurium* DT104, and *Staphylococcus aureus*) to levels as low as log 1 cfu/ml at 24 h (Anteneh et al., 2011). Likewise, the counts of *Sh*. *flexneri* and *S. aureus* co-cultured with LAB in *borde* reduced by greater than 1 log unit in 24 h (Girum et al., 2005b). In addition to these, the re-isolates of the probiotic additive from probiotic cheese containing *L. fermentum* strain ME-3 showed some decrease in antagonistic activity against *Shigella sonnei* ATCC 25931, *Staphylococcus aureus* B46, as compared with the original culture of ME- 3 according to (Songisepp et al., 2004). *Enterobacteriaceae* and coli-forms, microorganisms' indicative of the bacteriological quality of foods, were also detected at low levels (< 10^2 cfu/g) in hard cheese produced from *L. rhamnosus* LC 705 (B) and *L. paracasei* ssp*. paracasei* DC 412 (C) at 24 h due to the increasing population of LAB (Kalavrouzioti et al., 2005).

The survival of *Sh. boyidii* in the presence of LAB isolates might be interpreted, due to the high resistance capacity of *shigella* at lowered pH (Adams and Moss, 2008). The ability of *Shigella* to produce acid from glucose according to Adams and Moss (2008) may be responsible for their survival at low pH. *Sh. flexneri* was reported to develop more tolerance to lactic acid than to other organic acids (Girum et al., 2005b).

Conclusions

This study demonstrated that *ayib* enriched with potential inhibitory LAB possesses antibacterial effect against *S. aureus* ATCC 25923*, Sh. boydii* and *Ps. aeruginosa* ATCC 25853 with the highest effect against *Ps. aeruginosa.* The results indicate the possible use of these LAB isolates with potential probiotic property (at least to some *in-vitro* test) as biopreservatives against spoilage

and foodborne pathogens in *ayib*.

The LAB isolates with probiotic potential not only showed to improve the safety of *ayib*, but also found extending the keeping quality of the same local fermented product. Considering the impact of mixed cultures and longer survival rate of the LAB strains in the products, this study suggests that the isolates are possible good candidate starters for preparation of *ayib*; and moreover, *ayib* can be employed as vehicles for provisions of these potential health promoting strains.

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Conflict of interest

The authors declare that there is no conflict of interest among them.

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