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Review

Fusarium spp. and fumonisin in feed for equine and its importance for occurrence of leukoencephalomalacia

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In the animal feed industry, an ever growing increase in quality has been observed, but the main feature of a food is related to its security because the contamination poses a risk to animal health. The problems caused by fungal colonization is the significant loss of food quality, because even those who do not produce mycotoxins cause losses in the nutritional quality. Researches show that the most important Fumonisins are the mycotoxins found in corn, particularly when cultivated in warmer regions, produced by the fungi *Fusarium verticillioides* and *F. proliferatum.* Equine leukoencephalomalacia is a disease caused by ingestion of mycotoxin produced by the fungus *F. verticillioides.* Those infective mycotoxins are fumonisin (B1, B2, A1 and A2), having the B1 type as the most common and the most severe. The animals contamination occurs by ingestion of corn and its by-products in food that are contaminated by the fungus. This review addresses the importance of fungal contamination of the genus *Fusarium* by the production of fumonisin in horse feed and its relation to leukoencephalomalacia.

Key words: Fungi, mycotoxins, corn, food.

INTRODUCTION

Horses are animals that have been historically used by humans, mainly as a means of transport and also as an instrument of war. Over the years, horses developed skills ranging from physiological and physical features, until achieving the current conformation presented nowadays (Santos et al., 2012).

The human's relationship with horses is reported since the beginning of domestication, involving various functions like riding cavalry, some work activities and also used for leisure (Vieira, 2012).

With the development of management, sought to increasingly attend the nutritional needs and the welfare of these animals, deploying horse breeding, which has conquered several other areas of horse action, which involves leisure activities, sports and therapy with these animals (Santos et al., 2012).

In Brazil, the horse breeding has been outstanding and has been developing research and practices in nutrition

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of these animals, which has been serving the needs and optimizing the functioning of the competitive market of sport horses in the country (SÁ, 2014). In Brazil its main functions still remain in the work of agricultural activities, which basically involve the management of cattle (MAPA, 2014).

The world population of horses is estimated in 59.8 million of heads, which is a stable number since 2010 (FAO, 2013). In Brazil, much of the herd is in the Southeast, then immediately appear the Northeast, Midwest, South and North. It can highlight the Northeast, because of having the largest concentration of horses, and more registration of asses and mules (MAPA, 2014). Horses are animals with nutritional requirements that are basically determined by the maintenance of power-up and also for its energy that is used to perform physical activities, thereby determining the feeding of those animals must be balanced and must be a composition proper proportions of nutrients, which determine maintenance of your body condition. The amount of food that a horse can eat in your daily diet will depend on certain requirements, among them, the dry matter content of food relative to their body weight, their performance, and also relating to their physiological state and level of physical activity performed (Ribeiro et al., 2009).

Regarding its food habits and physiology, horses are classified as monogastric animals, vegetable grazing, and have the characteristic of food selection capability where they choose more often the leaves, stems and buds. They spend in grazing about 10 to 16 h a day, their meals are on average 2 to 3 h and in some cases have short rest intervals (Dittrich et al., 2010).

In the animal feed industry seeing an increasingly growing increase in quality, and it relates to various parameters relating to the constitution of food, the balance, palatability, digestibility and acceptability of such products for animals. However, the main feature of a food is linked to its security because the contamination poses a risk to animal health (Góes, 2011).

Equine leukoencephalomalacia (ELEM), also known as equine mycotoxic encephalomalacia or corn poisoning is a devastating neurologic diseases of equidae characterized by acute central neurological clinical signs associated with liquefactive necrosis of the cerebral sub-cortical white matter. The disease has been reported in several countries and it is caused by ingestion of one or more type of fumonisins (Kellerman et al. 1990), mycotoxins produced by several species of fungi of the genus *Fusarium*, including *F. proliferatum* and *F. verticillioides* (formerly *F. moniliforme).* Of these, fumonisins B1 (FB1), B2 (FB2) and B3 (FB3) are the most common in nature and FB1 is the most frequently detected in corn worldwide and the most commonly associated with ELEM outbreaks. Fumonisins are responsible for a variety of health problems in several animal species, including humans (Shephard et al., 1996; FDA 2001).

REVIEW

Fungi in animal feed

Fungi are characterized by being microorganisms that have wide distribution in the environment, with high geographic dispersion. They are important in several economic activities such as production of food, drugs, enzymes and organic acids. Other aspect of these organisms is that some fungi are pathogenic to plants and food spoilage, which may cause reduction in the nutritional value of food, the production of secondary metabolites and diseases in humans and animals (Silva et al., 2015).

The fungi naturally disperse through the atmospheric air. This is the way most used by fungi, by their spores and vegetative mycelium fragments that are released into viable portions of these organisms for air dissemination process. The fungal conidia are very important in this spreading segment, as the biological material suspended in the air often becomes imperceptible in an epidemiological analysis (Lobato, 2014).

The process of plant infection with the fungus is caused by the pathogen contact with the host root, so the plants can germinate resistance structures, stimulated by exudates produced by the plant and this determines a prominent infection. The penetration process occurs through the primary root or rhizoid and also absorbent structures. The infection process by the fungus, descriptively is performed through wounds or natural openings in the cell wall of roots, the effectiveness of this infection and subsequent development in the host includes the production of enzymes, toxins and virulence factors (Gawehns et al., 2014).

The colonization occurs with the intercellular growth hyphal toward the xylem vessels. As a result, the development of the fungus will lead to the obstruction of vessels due to the accumulation of structures and substances formed by the pathogen. In this intrinsic process, some mycotoxins can be produced by the fungus, and can naturally also block the vessels, and its damaging effects can destroy surrounding cells, causing accumulated material reaching the leaves. It also reduces the synthesis of chlorophyll, decreased movement of water and nutrients rate, which results in further decrease in cellular respiration as a result harmful effects on the production of the fruit (Takken and Rep, 2010).

It occurs a blackening of the vessels caused by substances that result from oxidation and polymerization of phenolic compounds. The symptoms spread throughout the plant leaves and, depending on the aggressiveness of some isolated, they can quickly become necrotic (Ma et al., 2013).

After infection by pathogenic fungi, some forage grasses show biochemical changes that lead to a loss of nutritional quality, and consequently also the food palatability for animals due to the reduction in the

concentration of proteins, amino acids, sugars, soluble carbohydrates and digestibility of the dried matter, and increased phenolic compounds and lignin in the infected plants (Martinez et al., 2010).

The problem caused by fungi colonization is the significant loss of food quality, even those who do not produce mycotoxins can cause losses in nutritional quality for being heterotrophic and they are not able to produce their own food, needing of nutrients present in the substrate inhabited (Gimeno and Martins, 2011). Fungi that produce mycotoxins include in the grain and make use of the substrate as a source of energy and nutrients and require a minimum moisture content of the substrate between 13 and 25%; relative humidity of the air greater than 70%; the presence of oxygen and the presence of appropriate temperatures to make their development in infested crops (Alfonzo et al., 2011). The fermentation, coloration changes, staining, changes in the odor and flavor, chemical changes, loss of dry matter, decreased germination and production of mycotoxins are the major damages to stored grain because of the fungi development (Ferrari Filho, 2011).

Contamination of raw materials directly affects livestock productivity and contributes to the loss of crops, which causes economic losses to the food industry and costs for control and analysis of mycotoxins (Cheli et al., 2013). In the infections caused by fungi in animals, pathogens establish infection opportunistically invading host tissue as soon invades the immune system, making survival mechanisms, then they can reproduce in this environment. To survive fungi go to nourish in infected tissues, and in cases of systemic infection, they spread to new tissues or organs (Negri et al., 2012).

Fusarium

The *Fusarium* is cosmopolitan fungi, which are largely inhabitants of the soil and is found in more favorable conditions in temperate and tropical regions. Many species are pathogens of cultivated plants, especially those that are important in the agricultural sector (Leslie; Summerel, 2006).

The genus *Fusarium* belongs to Nectriaceae family, Ascomycota phylum, and this fungal genus was described and classified for the first time in 1809 by the German mycologist Link (Maciel, 2012). Among the duties of the genus *Fusarium*, one is having wide geographical distribution, and its dispersion and development factor in the environment may be associated with the types of the climate, the vegetation, the microbiota, the type of soil and nutrients. It is also distinguished by having rapid growth, colonies with pale and colorful colorings and aerial and branched mycelium (Maciel, 2012; Frias, 2014).

Approximately about 1000 *Fusarium* species were described in 1900, based primarily on structure tests, including sporodochia in plant materials analyzed. These large number of species have been reduced by Wollenweber and Reinking (1935) in 65 species, 55 varieties, 22 forms, and arranged in 16 sections (Leslie et al., 2013).

The importance of this fungus is evidenced by most of these species are plant pathogens and widely inhabit the soil (Guarro, 2013).

In this genus the spores have two forms that are called microconidia and macroconidia. Microconidia are unicellular, uninucleate and fusiform. The macroconidia are multicellular, but each cell has only one core (Sandoval, 2010).

Pathologies triggered in cultures by the *Fusarium* species has as a imminent result the rotting of the roots, stems and fruits (Menezes et al., 2010). In corn culture, *F. verticillioides* and *F. graminearum* are the main pathogenic species of *Fusarium* that can cause various diseases associated with drastic reductions in productivity result in grain quality (Kuhnem Júnior et al., 2013).

In general aspects, the genus *Fusarium* is identified as a fungus that can be potentially pathogenic to plants, animals and humans and can also be a producer of secondary metabolites which cause poisoning by ingestion of food contaminated by humans and other animals (Leslie et al., 2013).

Mycotoxins

Mycotoxins are products that result from fungal metabolism. That is, they are secondary metabolites that can affect both human and animal health. Typically, mycotoxins are present in the environment in which they develop, such as grain-based food, cereals and feed. The environmental factors that contribute to the occurrence of mycotoxins are mainly ambient temperature, high humidity of the substrate associated with the processing, production or storage, and food type (Ferreira, 2012).

Mycotoxins cause pathological and functional changes in the animal body, which are called mycotoxicosis. One of the main damage caused by mycotoxins is its carcinogenic effect, which can affect animals and humans (Pereira; Santos, 2011).

The presence of fungi in agricultural products does not mean that the fungus produced mycotoxins. However, the detection of mycotoxins can occur with no presence of the fungus in food, since mycotoxins are highly resistant to adverse conditions such as industrial processes involving mechanical and thermal phases. In this way, the mycotoxins may remain in food even after the elimination of the fungus that produces them (Oliveira and Koller, 2011).

Mycotoxins can cause various harmful biological effects in the animal organism, including, hyperestrogenism, nephrotoxicity and hepatotoxicity (Rocha et al., 2014). The establishment of strict limitation and tolerance levels

Table 1. Permitted limits for mycotoxins in various species.

Mycotoxin	Feed stuff(s)	Limit (ppb)	Country / Authority
Aflatoxin B1	Maize	5	Turkey, Russia, Egypt.
	Maize	10	China, Korea, Japan
	Animal feed	10	Egypt
	Animal feed	50	Turkey
	All cereals except rice and maize	2	EU
	Unprocessed maize and rice	5	EU
	Animal feed ingredients	20	EU
	Feed stuffs for immature animal	20	FDA
Aflatoxin B1& G1	Maize	30	Brazil
Aflatoxin M1	Milk	0.5	U.S.A, Russia, Egypt
		0.05	Turkey
	Milk and milk products	0.05	EU
	Milk	0.5	FDA
Deoxynivalenol	Unprocessed cereals other than wheat, oats and maize	1250	EU
	Unprocessed wheat and oats, maize	1750	EU
	Cereal products	500	EU
	Cereals and cereal products for feed	8000	EU
	Maize by-products for feed	12000	EU
	Animal feed	100	FDA
Fumonisin B1, B2	Animal feeds except Equines	50	EU
	Animal feeds for Equines	5	EU
Fumonisin B1, B2, B3	Animal feeds except Equines	30	FDA
	Animal feeds for Equines	5	FDA
Fumonisins	Unprocessed maize	2000	EU
	Maize products for human	1000	EU
Ochratoxin A	Unprocessed cereals	5	EU
	Cereals and cereal products for feed	250	EU
	Cereal products for food	3	EU
T-2 and HT-2	All cereals grains	100	EU
Total aflatoxin	Animal feed ingredients	50	EU
	Animal feed	20	Canada, Egypt, Iran
		50	Brazil
	Maize	10	Turkey, Egypt
		30	India
	Cereals feedstuffs	200	Mexico
	Feedstuff (ingredient)s	20	Japan, U.S.A, Korea
	All cereals except rice and maize	4	EU
	Maize and rice	10	EU
	Feed stuffs for mature animal	100	FDA
Zearalenone	Unprocessed cereals other than maize	100	EU

EU: European Union. FDA: Food and Drug Administration. Source: Abdallah et al. (2015).

of mycotoxins is held by national and international authorities such as the European Commission (EC), US Food and Drug Administration (FDA) and World Health Organization (WHO) as shown in Table 1. FDA has established the maximum acceptable limits in food for sum of AFs (B1, B2, G1, and G2) at 20 μg/kg and AFM1 in milk at 0.5 μg/kg while the total Afs residue limit in feeds for mature and immature animals is 100 and 20 μg/kg, respectively (Womack et al., 2014). Up to date, the major source of food and feed all over the world is cereal grains.

As a result of their health implications and increasing knowledge of health hazards, regulations for major mycotoxins in commodities exist in at least 100

Table 2. Toxic effects of mycotoxins in different animals.

Mycotoxin	IARC† classification	Major effects	Clinical and pathological signs on most susceptible animals
Aflatoxins Aflatoxin M1	1 2B	Carcinogenic, hepatotoxic and impaired immune system	Reduced productivity; inferior egg shell and carcass quality; increased susceptibility to infectious disease.
Ochratoxin A	2B	Carcinogenic, nephrotoxic, hepatotoxic, neurotoxic and teratogenic	Kidneys are grossly enlarged and pale due to nephrotoxicity; fatty livers in poultry; shell decalcification/thinning.
Deoxynivalenol	3	Immunotoxic and ATA (alimentary toxic leukopenia)	Decreased feed intake and weight gain in pigs; feed refusal and vomiting at very high concentrations.
Other trichothecenes (T-2 toxin)	3	Immuno-depressants, gastrointestinal haemorrhaging and hematotoxicity	Reduced feed intake; vomiting, skin, and gastrointestinal irritation; neurotoxicity; abnormal offspring; increased sensitivity to infection; bleeding.
Zearalenone	3	Fertility and reproduction (estrogenic activity) and disrupts endocrine system	Swollen, reddened vulva, vulvovaginitis, anestrus vaginal prolapse and sometimes rectal prolapse in pigs; feminization and suppression of libido; suckling piglets may show enlargement of vulvae; fertility problems.
Fumonisins	2B	Carcinogenic, hepatotoxic, central nervous system damage and immuno-depressants	Equine leucoencephalomalacia (ELEM), porcine pulmonary edema, liver damage in poultry.

†International Agency for Research on Cancer. 1: carcinogenic to humans AFs; 2A: probably carcinogenic to humans; 2B: possibly carcinogenic to humans; 3: not classifiable as to its carcinogenicity to humans; 4: probably not carcinogenic to humans. Source: Abdallah et al. (2015).

countries (Oruc et al., 2006; Cheli et al., 2014). Because of the health risks of toxicity, Brazilian law establishes maximum levels for AFB1 (5 µg kg^{-1}) and OTA (10 µg kg^{-1}) in cereals and cereal products; and ZEA e in wheat flour and bakery products (200 µg kg^{-1} until December of 2015, and from January of 2016 this limit will be reduced to 100 µg kg^{-1}) (BRASIL, 2011).

The thresholds of toxicity of mycotoxins intake by equine species vary from animal to animal and depend on parameters such as their health status, level of work and the reproductive stage of this species. The mycotoxins sporadically occur in isolation and its effects are usually synergistic or cumulative. In the body of the equine species, the mycotoxins ingested in the feed are absorbed before occurs post-gastric fermentation. The metabolites reach the small intestine where they exert their effect on the intestinal wall. After, they are absorbed and distributed via the bloodstream (Knowmicotoxins, 2015).

The factors that provide or interfere with fungal growth and production of mycotoxins are physical, chemical and biological. Physical factors are moisture or free water, water activity, relative humidity, temperature, microflora zones, and physical integrity of grain. Chemical factors are the pH, substrate composition, minerals and nutrients, redox potential (O_2 / CO_2). Biological factors are characterized by the presence of invertebrates and specific strains with production ability (Gimeno and Martins, 2011).The mechanism of action of mycotoxins in the host involves the impairment of the animal's immune status that favors various infections, which will depend on the type of mycotoxin involved (Table 2). These attributes are a major reason for the difficulty of diagnosis of mycotoxicosis (Iheshiulor et al., 2011).

Six classes of mycotoxins are considered the most significant in agriculture and in the food industry: aflatoxins (AFs), ochratoxins (OTs), fumonisins (FBs), zearalenone (ZEN), deoxynivalenol (DON) and other trichothecenes, and Patulin (Figure 1). They are the most widespread mycotoxins in animal feed and human food (European Food Safety Authority, EFSA, 2010).

Fumonisin

The fumonisins had its first statements about 1988. They are produced by the gender *Fusarium*. The main species that produce fumonisins are: *F. verticillioides, F. proliferatum, F. nygamai, F. anthophilum, F. dlamini, F. napiforme, F. subglutinans, F. polyphialidicum* and *F. oxysporum* (Cruz, 2010; Mallmann et al., 2013).

There are several types of fumonisin due to the large amount of producing species. So far, it is known around 25 substances, which B1, B2 and B3 occur more frequently in food (Cruz, 2010; Pereira and Santos, 2011; Santana, 2012).

The climate in Brazil favors the contamination of grain by fungi. The conditions of high humidity and temperatures of about 20 to 26°C are optimal for the production of these metabolites (Cruz, 2010). In Brazil there is a high incidence of contamination
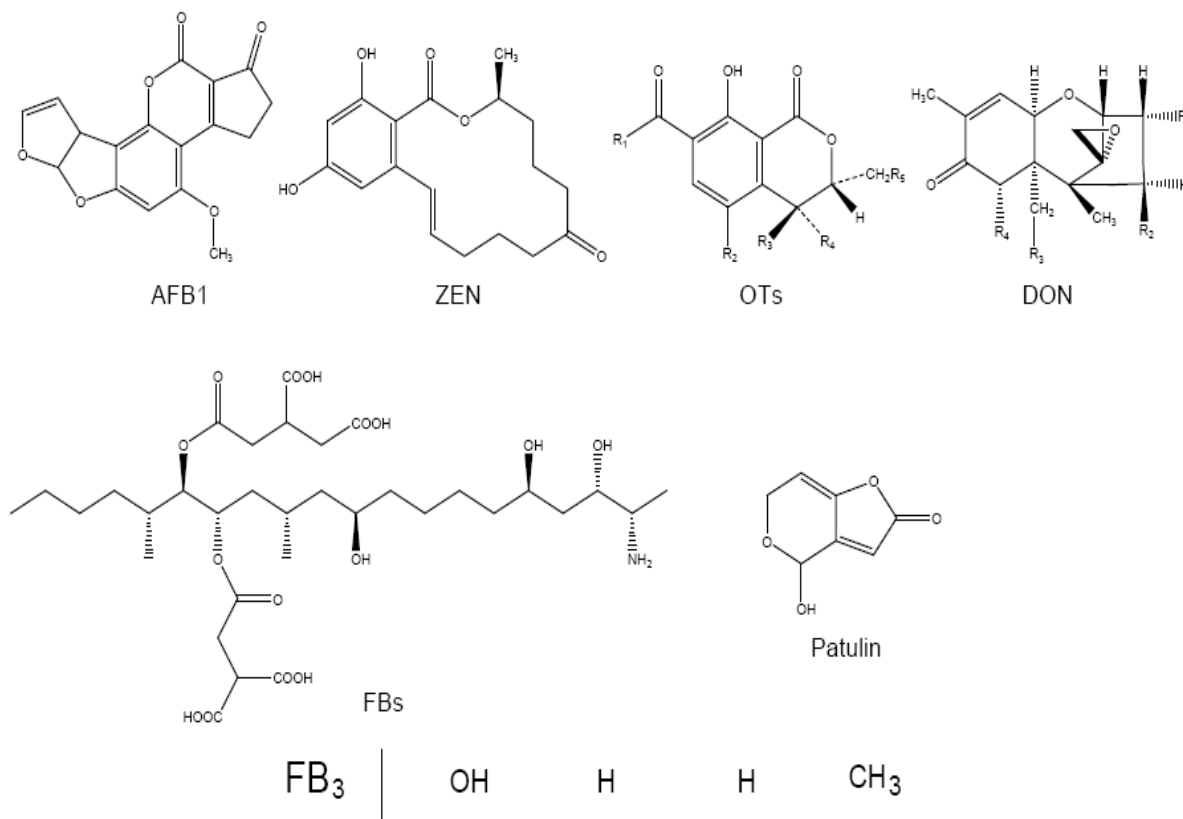
Figure 1. Chemical structures of some of the major mycotoxins produced by *Fusarium* spp.

by fumonisins in diets in general. The raw material of these feeds, especially corn and its derivatives, are naturally colonized by these fungi producing mycotoxins. This condition can lead to a high incidence of contamination in feed intended for animal consumption. (Souza et al., 2013; Cardoso Filho et al., 2015). This natural contaminant of cultures occurs worldwide and is of great importance for the economy and public health (Marin et al., 2013).

Studies report that diseases caused by fumonisin are quite frequent. The chronic use in animal of subclinical levels of toxin can degrade the function of the immune system (Grenier et al., 2011).

Fumonisin is highly toxic and is found not only in corn and its derivatives, but it can be found in many other types of foods such as beer, brewer's grains, wine, rice bran, sorghum, millet or folder. The metabolites may occur in low concentrations in a production. This metabolite often is linked to internal array of food, and it is not easily extracted due to its strong interaction, non-covalent, with macromolecules of food matrix (Scott, 2012).

The toxic mechanism of Fumonisin B1 in the animal organism is related to inhibition of biosynthesis of sphingolipid of cell membrane. Such inhibition can cause several cellular damages. Parallel pathways may be affected by the inhibition of ceramide synthase, as well as the bioactivity of sphingoid bases and their metabolites, and the multiple functions of more complex sphingolipids (Merrill Jr. et al., 2001; Soriano et al., 2005).

This is because fumonisins have similar structures to the precursors of sphingolipids, which allows change of important cellular functions, such as control of membrane integrity, cell proliferation, differentiation and apoptosis (Rocha, 2014).

Acute and chronic effects of toxicity of FB1 include diseases such as esophageal cancer and neural tube defects in humans. It can cause carcinogenicity, hepatotoxicity, nephrotoxicity and neurotoxicity in animals (Rocha et al, 2014).

Analytical methods for the determination of fumonisin are typically based on chromatography techniques (high-performance liquid chromatography) in combination with a variety of detection methods (molecular fluorescence or mass spectrometry) (Shephard et al., 2012).

Research has shown that the Fumonisins are the most important mycotoxins found on corn, especially when grown in warmer regions. The producing fungi, such as *F. verticillioides* and *Fusarium proliferatum*, can grow over a wide range of temperatures, but only with relatively high amounts of water. (Cao et al., 2013; Cardoso Filho et al., 2015).

Equine leukoencephalomalacia

Equine leukoencephalomalacia is a disease caused by ingestion of mycotoxin produced by the fungus *F. verticillioides*. The infective mycotoxins are fumonisins B1, B2 and B3. The mycotoxin B1 is more frequent and severe. Animal intoxication occurs by ingestion of corn and its sub-products contaminated by the fungus (Del Fava, 2010).

The use of corn and its derivatives in the feeding and supplementation of equine diet is needed during the shortage of pasture forage (Del Fava, 2010). In a retrospective study of equine diseases in Rio Grande do Sul-Brazil, leukoencephalomalacia was the nervous system disease most frequent (Pierezan, 2009).

Fumonisins (B1, B2, and B3), in the central nervous system equine, develop sudden neurological signs due to the liquefaction necrosis of the white matter. The animal's death may occur 4 to 72 h after the clinical course. However, depending on the amount of infective dose of mycotoxins, animal survival time can extend from one to two weeks (Méndez; Riet-Correa, 2007).

The use of corn-based supplementation in the diet of equine involves products such as natural corn, pollard, bran or corn xerém. Some regions make use of waste processing industries of this grain, particularly during the shortage of fodder in pastures, which favors the onset of disease (Ragsda and Debey, 2003).

Frequently detected in corn worldwide and the most commonly associated with leukoencephalomalacia outbreaks. Fumonisins are responsible for a variety of health problems in several animal species, including humans. These compounds are carcinogenic in laboratory rodents and the International Agency for Research on Cancer of the World Health Organization has included them in the list of probable carcinogenic substances for humans. Amongst the domestic animals, horses are the most sensitive to fumonisin intoxication, the toxic effects of FB1 in this species being dose-dependent. The clinical signs include decreased tongue tone and mobility, propioceptive deficit, ataxia, anorexia, lethargy, blindness, circling, aimless walking, head-pressing, hyperexcitability, diaphoresis and coma. Affected animals that develop clinical signs but survive usually show some degree of neurologic deficit for life (Maxie; Youssef 2007; Pacin et al., 2009).

The pathogenesis is not yet completely understood. The enzyme sphingosine-N-acyltransferase is structurally inhibited by fumonisins. This enzyme is involved in sphingolipids biosynthesis and it is hypothesized that the accumulation of the enzyme substrates as well as the depletion of complex sphingolipids, may account for the toxicity of these. The characteristic gross lesion is restricted to the white matter of the cerebral hemispheres and consists of softening, cavitation and yellow discoloration. The lesion may be focal or multifocal, uni or bilateral and mild cases may not show gross lesions at all. Histologically, the most characteristic lesions consist of areas of liquefactive necrosis, edema and hemorrhage affecting the encephalic white matter A presumptive diagnosis is established based on clinical signs and on gross and/or histological findings. Confirmation of the diagnosis relies on detection of toxic concentrations of fumonisins in feed (Beasley, 1999; Maxie and Youssef, 2007).

In horses, the signs are characterized by brain injuries, brain stem injury. In mules, the predominant signs are generally characterized by lesions of the brain stem (Riet-Correa et al., 2007).

PREVENTIVE ACTIONS

The use of pasture rotation in non-host plants and the elimination of crop residues are ways that favor the decrease in pathogenic strains. The increase in microbial activity is decreased by effective practices that increase soil supressiveness by the antagonist ability of these methods provide against potential pathogen (Zhao et al., 2013).

The use of integrated management in modern agriculture has contributed in order not only to increase production in less space, but also in significant improvement in the control of weeds, pests and diseases. The main advantages are increased productivity of crops, increased profitability, significant reduction in production costs, rational use of pesticides, reduced use of water and fuel, and less use of machines for cultivation which leads to lower release of greenhouse gases (Lerayer, 2010).

Advances in crop technologies have increased the productivity of maize. Examples of these new techniques are the direct planting, which uses correction and proper soil fertilization; extensive use of integrated management techniques of weeds, diseases and insect pests; and increased adoption of improved seeds with high production capacity. The most important contributions in the use of these new techniques are the use of simple hybrids and adoption of genetically modified seeds (Gravina; 2011).

Some *Fusarium* spp. tend to be less aggressive, which can be observed by analysis of visible symptoms in the host. Some of these strains can be represented by *F. verticillioides*, which nevertheless is an excellent pathogen in the production of mycotoxins. However, these pathogens should not be neglected since the resistance of a species cannot be extended to the others. That is, resistance to an isolated pure from one species can not result in cross-resistance to a *Fusarium* population in commercial corn fields (Reid et al., 2009). The conditions for fungal growth and therefore mycotoxin production depends on environmental factors and erroneous parameters, such as agricultural production without technical and preventive measures, inadequate drying, handling, packaging, storage, and transport conditions that may promote fungal growth (Marin et al., 2013).

In general, fungal infestations are difficult to be handled by conventional methods due to the ability of these pathogens to survive in different environments. Among these places, the soil and crop residues can be cited, which characterizes the persistence of these pathogens. An efficient and cost-effective control technique is the use of resistant cultivars. (Bakhsh et al., 2007).

The fungicides carbendazim, thiram + benomyl, and thiram + captan, are used for seed treatment (Nene et al., 2012). The systemic use of chemical fungicides on plants is considered costly because it may bring about damage to the environment. Another problem is that these fungicides cannot prevent infection and colonization of roots by the pathogen (Animisha et al., 2012).

The preventive measure against poisoning of equines is based on the recommendation of the use of corn and its sub-products in amounts lower than 20% of total of dry matter ingested by these equines. The corn used must be subjected to a correct drying process. However, the disease can occur in equines who eat corn dried previously with moisture within the standards required in Brazil, which is less than 15%. Thus, it is necessary to completely dry so that there is no possibility of fungal growth in the raw material (Méndez and Riet-Correa, 2007).

CONCLUSIONS

Thus, it can be noted that the equine feeding with the use of ration requires a lot of care. Such care may range from preparation of feed until the supply of the animals. The processes of production, storage and delivery when not held properly could favor the growth of fungi, such as those from the genus *Fusarium*. Fungi of this genus are producers of fumonisin, which can lead to leukoencephalomalacia outbreaks, among other pathologies. Therefore, it is emphasized the importance of control methods and awareness for the production of feed. These actions may help to minimize the contamination by fungi and reduce the risk of diseases to equines, what may lead to a better nutritional quality for the animals and less economic losses.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Microbial species of safety concern in milk from informal processors in Harare, Zimbabwe

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In this study, the bacteriological quality and the presence of *Staphylococcus aureus*, *Escherichia coli* O157:H7, *Bacillus cereus*, *Salmonella* species, and *Pseudomonas* species were studied in raw milk, cultured milk, milk handlers and packaging containers. A total of 36 samples were collected over 3 months from three different farmers. Samples were analyzed for means of counts per milliliter of milk for total bacterial count (TBC), total coliform count (TCC), total *E. coli* count (TEC), *S. aureus*, *B. cereus*, *Salmonella* spp. and *Pseudomonas* spp. Microbial load ranged between 0.81 and 7.6 $\log_{10}$ cfu/ml for various critical sampling locations. Isolates of *E. coli*, *S. aureus* and *B. cereus* were taken for simple polymerase chain reaction (PCR) to investigate the presence of virulent genes, *rfB*, *sei*, and *cytK* with amplicon sizes of 1.0 kb, 500 bp and 320 bp, respectively. The *sei* gene was detected in 19% of the samples and 2.8% were found to have the *cytK* gene. The *rfB* gene could not be picked in *E. coli*. The results show poor hygienic practices at the processors and potential risk to the consumers.

Key words: Fresh milk, fermented milk, packaging container, milk handler, pathogens.

INTRODUCTION

The occurrence of several serious food safety problems in the last decade has put higher demands on the control and assurance of food safety and quality by all actors in the agro-food chain (Jacxsens et al., 2010). Although other dairies have made significant efforts and investments in designing and implementing good hygiene practices, using hazard analysis and critical control point (HACCP) based systems (CAC, 2003) the dairy industry

is still faced with the challenge of implementing good hygiene practices to ensure the production of safe products especially in the developing countries (Milios et al., 2012; Kussaga et al., 2014). While it has been reported that fresh drawn milk from a healthy cow normally contains a low microbial load (<3 $\log_{10}$ cfu/ml), the load may increase by 2 $\log_{10}$ cfu/ml, or more, once it is stored at room temperature (Walstra et al., 2005;

Bytyqi et al., 2011). Several studies have reported the presence of both gram positive and gram negative pathogens in the dairy industry (Amagliani et al., 2012; De Vliegher et al., 2012; Gurol et al., 2012; Lee et al., 2012, 2014; Ranieri et al., 2012; Barancelli et al., 2013). Control activities should therefore be instituted to prevent microbial contamination and growth in production areas thereby reducing pathogens (Luning et al., 2008; Jacxsens et al., 2010; Milios et al., 2012).

Consumption of unprocessed milk and its by-products is potentially hazardous and has been associated with several types of infections including brucellosis (Ramos et al., 2008), tuberculosis (Doran et al., 2009), salmonellosis (Poppe, 2011), yersinosis (Greenwood and Hooper, 1990), *Escherichia coli* O157:H7 (Anand and Griffiths, 2011) and staphylococcal enterotoxins poisoning (Baylis, 2009; Ostyn et al., 2010). Despite modern dairy ensuring that consumers get processed safe milk, consumption of unprocessed milk is still common in Zimbabwe especially in the rural areas with the two main types being fresh liquid milk and naturally fermented milk. Although the food regulations in Zimbabwe impose a general responsibility to ensure safety of any food brought to the market on the producer, this has not always been the case due to a number of factors including, cultural, economic and poor legislation enforcement among others.

The Zimbabwean dairy industry can be easily categorised into 2 groups, the formal and the informal sector with the latter posing greater safety concerns. The informal dairy sector mainly produces two products, raw milk termed "fresh milk" and the naturally fermented/cultured milk (Amasi/Hodzeko). The latter product taking greater preference, because it is used as a relish which provides proteins at a low cost for the lower class while it is a delicacy for the affluent that they consume as part of the main meal or dessert. Due to the faltering economy of Zimbabwe, many dairy farmers have been downsizing operations and ultimately preferring to sell their milk product directly to consumers without supplying any processor (Gadaga et al., 1999; Gran et al., 2003).

Fermentation of milk is important as it is a cheap traditional way of improving nutritional properties as well as sensory properties (Gadaga et al., 1999). It is not only effective on flavour development but has been used as a preservation method for many years; hence, fermented foods are regarded as microbiologically safe. The low pH is effective in inhibiting the growth of many microbes (Jeevartnam and Jamuna, 2004). *Staphylococcus aureus* among other pathogens is destroyed by pH lower than 5 (Medveďová and Valík, 2012). However, emerging research has shown that some strains of *E. coli* can survive the low pH (Gran et al., 2003; Bore et al., 2007). A combination of harsh conditions and prolonged exposure time has been said to result in microbes building some adaptation mechanism (Lucking et al., 2013). The ability of pathogens such as *E. coli* to survive

low pH emerges as a potential health hazard to consumers of the naturally fermented milk, which is generally regarded as a safe product in Zimbabwe due to its low pH. The aim of this research was to determine the incidence of pathogens (*E. coli, S. aureus,* and *Bacillus cereus*) in fresh milk, naturally fermented/cultured milk, milk handlers as well milk packaging containers used at farm level in the informal sector in Zimbabwe.

MATERIALS AND METHODS

Three farms (FD 1, FD 2 and FD 3) around Harare province were selected for the research. The microbial assessment scheme (MAS) methodology developed by Jacxsens et al. (2011) was used to determine the food safety status in the informal milk processing farms. A total of 4 critical sampling locations (milk handler, milk container, fresh milk, and cultured milk) were selected. A total of 4 samples (milk handler swab, milk container swab, 50 ml fresh and cultured milk) from each dairy farm were collected during each visit and the sampling was done 4 times over a three month period. The samples were transported at 4°C to Government Analyst Laboratory for analysis within 3 h of collection. Samples were analyzed for total aerobic bacteria count (TBC), total coliform count (TCC), *E. coli, Salmonella* species, *Pseudomonas* species, *S. aureus*, and *B. cereus*. Methods of microbial analysis are summarized in Table 1. After enumeration, isolates were, purified and stored at -20°C in 30% sucrose solution awaiting polymerase chain reaction (PCR).

PCR Reactions

B. cereus, S. aureus and *E. coli* isolates were resuscitated and DNA was extracted according to procedures described in Medina-Acosta and Cross (1993). All PCRs were carried out in 10 µl reaction tubes in a Bio-Rad T100™ Thermal Cycler (Singapore). The reaction mix consisted of 1 µl bacterial DNA template, 5 µl KAPA BIOSYSTEMS 2X KAPA Taq Ready Mix (Cape Town, South Africa), 3.4 µl PCR-grade water, and 0.6 µl primers. The primers and annealing temperatures used in the reactions are listed in Table 2. The PCR conditions for *cytK* are described in Swiecicka and Mahillon (2006), while conditions for *rfB* and *sei* are described in another study (Thapa et al., 2012). PCR products were separated by Gel electrophoresis using a 2% Agarose gel ULTRAPURE™ (USA) and the image was viewed using a Bio-Rad Gel Doc™ EZ (USA).

Statistical analysis of data

Descriptive statistics and other explanatory analysis were used as well as statistical techniques: univariate analysis (one way analysis of variance [ANOVA]) using SPSS version 16.0 and STATA 12 adjusted for multiple comparisons to detect significance difference between means. The significance test was at alpha level 0.05 or 95% confidence level.

RESULTS

Total bacterial count (TBC)

The average (TBC) for three batches was found to be low

Table 1. Microbiological analysis methods used to enumerate and isolate bacteria.

Organism	Media	Manufacturer	Incubation temperature (°C)	Incubation time (h)	References
Total bacterial count	Plate Count Agar (PCA)	Oxoid Basingstoke Hampshire, England	25, 30, 55	24	Duncan et al. (2004)
Total coliform	MUG Violet Red Bile (VRB)	Oxoid Basingstoke Hampshire, England	37	24	ISO 4832:2006
B. cereus	Tryptone Soya Agar (TSA)	Biolab, South Africa	37	24	ISO 7932:2004
E. coli	Tryptone Bile X-glucuronide (TBX)	Oxoid Basingstoke Hampshire, England	44	24	Aijuka et al. (2015)
S. aureus	Baird-Parker	Biolab, South Africa	37	24	Aijuka et al. (2015)

Table 2. PCR primers for *B. Cereus*, *S. aureus* and *E. coli*.

Microorganism	Target gene	Sequence	References
B. cereus	*cytK*	GAAACGGGCGCTGTTATCTT TGCTGCTTACGCTCAAACGA	Swiecicka and Mahillon (2006)
S. aureus	*Sei*	CAGGCAGTCCATCTCCTGTA AAAGGCGTCACAGATAAAAACC	Thapa et al. (2012)
E. coli	*rfB*	TAAGTAATGGAACGGTTGCTCT CCCCACTCGTAAAATCCATC	Thapa et al. (2012)

at FD 1 where it was ranging from 3.61 to 5.57 $\log_{10}$ cfu/ml in packaging container and fresh milk, respectively. Generally, the TBC in packaging containers was found to be lower than in milk handler, fresh milk and cultured milk in all the three dairies. The highest TBC was recorded in cultured milk from FD 2 and 3 which had 7.3 and 7.6 $\log_{10}$ cfu/ml, respectively. There was no change in levels of TBC from batch 1 to batch 3 during the sampling period. This is supported by an average of 3.49 $\log_{10}$ cfu/ml in milk handler batch 1 to 5.73 $\log_{10}$ cfu/ml in milk handler batch 3. Batch 3 had the highest average of TBC in all the three CSLs, ranging from 5.0 to 8.3 $\log_{10}$ cfu/ml.

Total coliforms

FD 1 had the highest coliforms ranging from 1.56 to 6.22 $\log_{10}$ cfu/ml in packaging container and fresh milk, respectively. FD 2 had the lowest coliforms, ranging from not detected in milk handler to 3.94 $\log_{10}$ cfu/ml in fresh milk. When comparing the growth pattern of coliforms in fresh milk and cultured milk, fresh milk had the highest number of coliforms.

Total *E. coli*

The milk handler from FD 2 and 3 had no *E. coli*

as well as the packaging container in FD 1. The highest number of *E. coli* was recorded in fresh milk collected from FD 1 and milk handler of FD 1. There was a gradual increase in *E. coli* in the fresh milk to cultured milk from FD 3. Fresh milk had 1.78 $\log_{10}$ cfu/ml and cultured milk had 2.21 $\log_{10}$ cfu/ml.

S. aureus

All three dairies were found to have *S. aureus* at all CSLs. The highest number of *S. aureus* was recorded in milk handler of FD 3 (4.4 $\log_{10}$ cfu/ml). The lowest was in the same dairy in cultured milk

with log 1.98 $\log_{10}$ cfu/ml. Packaging containers of FD 1 had high levels of *S. aureus* (4.18 $\log_{10}$ cfu/ml).

Pseudomonas spp.

The incidence of *Pseudomonas* spp. was generally low in all three dairies. However, the fresh milk collected from FD 1 had high levels of *Pseudomonas* (4.16 $\log_{10}$ cfu/ml) and the minimum recorded was 0.68 $\log_{10}$ cfu/ml. No incidence of *Pseudomonas* spp. was recorded in FD 3 in cultured milk and milk handler from FD3.

B. cereus

The highest incidence of *B. cereus* in all CSLs was recorded in FD3 followed by FD1. The least incidence was recorded in FD 2. Fresh milk in all three dairies had higher levels of *Bacillus* species when compared with cultured milks of all the three dairies.

PCR reactions

The genomic DNA for *S. aureus, B. cereus,* and *E. coli* were subjected to PCR to investigate the presence of virulent genes, *rfB*, *sei*, and *cytK* with amplicon sizes of 1.0 kb, 500 bp and 320 bp, respectively. The *sei* gene was detected in 19% of the samples and 2.8% were found to have the *cytK* gene. The *rfB* gene could not be picked in *E. coli.*

DISCUSSION

TBC results were not in agreement with previous studies (Al-Tarazi et al., 2003) who reported considerably lower levels of TBC in fresh milk with mean values of 1.1×10^7 cfu/ml. However, they concurred with previous studies in Zimbabwe that showed high TBC in raw milk (Gran et al., 2003). While it is reported that raw milk TBC should be less than 100 000 cfu/ml (Gosta, 1995), Zimbabwean food regulations require that raw milk should not exceed 20 000 cfu/ml (Food and Food Standard Act, 2001). High bacterial load in raw milk has been attributed to poor hygienic practices that include failure to properly clean the udder and use of contaminated water among others (Gran et al., 2003). The bacteria in milk handler are also likely to find its way into the raw milk and other subsequent stages of milk processing (Gran et al., 2003). In food safety, implementation of prerequisites and application of HACCP guarantee the control of processes (Domenech et al., 2013). The absence of documented hygienic practice followed by the processors in this study might also have contributed to high levels of contaminants. Cusato et al. (2014) also included diagnosis of prerequisites programs (PRPs), implementation of good

manufacturing practices (GMP), standard sanitation operating procedures (SSOP) and training of the food as the major steps in the implementation of food safety system. However, such conditions are likely to be found in formal organizations which are well established mostly with food safety management systems. Out of the fourteen PRPs (CAC, 2003; Jacxsens et al., 2010; Luning et al., 2008) informal processors in this study can concentrate on cleaning and disinfection, personal hygiene of the milk handlers, and temperature control as recommended by Kussaga et al. (2014) as a way of improving the microbiological quality of the milk. This concurs with Lee et al. (2012) who pointed out that high frequency of *S. aureus* isolates in dairy farms highlights the need for constant improvement of hygiene and quality assurance.

While fermentation results in increased acidity that discourages the growth of most pathogens (Gran et al., 2003), our results showed the presence of potential pathogens in both fresh milk and fermented milk. Our findings on coliforms and *E. coli* were in agreement with previous studies (Saudi and Moawad, 1990; Ahmed and Sallam, 1991; Sobeih et al., 2002; Al-Tarazi et al., 2003; Chye et al., 2004; Korashy and Mohammed, 2008; Altalhi and Hassan, 2009) who reported that all examined fresh milk samples were contaminated with coliforms. These results were in violation of both European and Zimbabwean standards for raw milk intended for processing and milk for consumption (Gran et al., 2003). These findings concurred with a previous study in Zimbabwe that concluded on poor hygiene and sanitary practices during milking and further handling (Gran et al., 2003).

In this study, fresh milk with 4.16 $\log_{10}$ cfu/ml of *Pseudomonas* spp. was found to be the most prevalent. The presence of *Pseudomonas* spp. in both fresh and fermented milk indicates that *Pseudomonas* spp. can survive in fermented foods. This is in violation of food regulations (Dairy Regulations, 1977; Food and Food Standards Act, 2001; Public Health Act, 2001; CAC, 2003) where the food is not supposed to have either pathogenic or spoilage organisms.

In spite of generally low *E. coli* counts, its presence is suggestive of poor hygienic indicators, faecal contamination and implies a risk that other enteric pathogens may be present in the product (Gran et al., 2003). The findings concur with previous studies which indicated that poor hygiene was evidenced by the presence of *E. coli* (Ghafir et al., 2008; Jacxsens et al., 2009; Sampers et al., 2010). The presence of *E. coli* violates regulations which prohibit the presence of pathogenic and spoilage microorganisms in any food item intended for human consumption (Dairy Regulations, 1977; Food and Food Standards Act, 2001; Public Health Act, 2001; CAC, 2003). The results in this study indicated that the *E. coli* survives in fermented milk. This concurs with another study which reported that acid adapted cells

showed a marked increase in levels of resistance to lactic acid even though the level of resistance varied among strains (Gregory et al., 1995). Our results showed that none of our strains belonged to O157:H7. This contradicted with a previous study that suggested the presence of the pathogenic *E. coli* in a number of food items (Prasad et al., 2012).

The presence of *S. aureus* at all the CSLs in all the dairies contravening various food regulations was in agreement with a previous study (Gran et al., 2003). The isolation rate observed in this study was higher than was reported by Abdel-Hammed and El Malt (2009) who reported that 24% were contaminated with *S. aureus.* Dissemination of *S. aureus* from humans to food can occur by direct contact, indirectly by skin fragments, or through respiratory tract droplet nuclei (Jablonski and Bohach, 1997). *S. aureus* is also commonly found in mastitis udder (Wellenberg et al., 2002), hence common in milk from animals suffering from mastitis. The minimum amount of *S. areus* required to produce intoxication in human is estimated to be about 5 $\log_{10}$ cfu/g (Rørvik and Granum, 1999). To produce sufficient enterotoxin, the pH should be higher than 4.6 and the temperature should be above 15°C for more than 3 to 4 h (Rørvik and Granum, 1999). If *S. aureus* gains access to the milk before fermentation, the pH would have been higher than 4.6 for longer than 6 h and therefore pose a definite risk of toxin production during the early part of the fermentation. Our findings on the presence of the toxin producing *sei* gene in *S. aureus* was in line with a previous study (Nazari et al., 2014), although we could not distinguish between the most toxic *sei* 1 and *sei* 2. Similar to previous studies (Larsen and Jogernsen, 1997), *B. cereus* was isolated in milk and other sampling locations. The prevalence of the *cytK* in *B. cereus* from our study was in line with previous studies that concluded that its distribution is low in *B. cereus*.

Conclusion

Fresh and fermented milk from informal processors in Harare pose a potential threat to consumers and this is in violation of both the Zimbabwean and international food regulations. Poor hygiene practices by the milk handlers maybe the greatest link of milk contamination. The presence of virulent genes (19% *S. aureus* and 2.8% *B. cereus*) confirms that consumers of fermented milk are at safety risk.

Conflicts of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Evaluation of a non-submerged cultivation assay combined to ESEM imaging for analysis of biofilms formed by dairy-associated sporeforming bacteria

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In the dairy industry, the biofilms formed by spore forming bacteria are not well characterized. Microscopic methods are crucial for the study of biofilm structural and architectural features. Here, a simple surface-associated non-submerged model combined to environmental scanning electron microscope (ESEM) imaging was used for the study of *Bacillus cereus* and *Geobacillus* spp. dairy biofilms. To evaluate the utility of this approach, non-submerged biofilms were compared to those developed *in situ* on stainless steel coupons introduced inside milk processing lines. Results reveal that both *B. cereus* and *Geobacillus* spp. are able to form specific biofilm characteristics on non-immersed surfaces, notably an original dispersion style not previously described. Non-submerged biofilms *in vitro* are elaborate three-dimensional or extensive complex structures well resolved in ESEM and comparable to dairy biofilms *in situ.* The non-submerged surface-associated biofilm combined to ESEM imaging revealed a relevant model for the study of dairy biofilms.

Key words: Biofilms, *Geobacillus*, *Bacillus cereus*, non-submerged surfaces, environmental scanning electron microscope (ESEM) imaging.

INTRODUCTION

In the dairy industry, the biofilms formed on equipment surfaces are recognized to be a major source of contamination of processed milk and dairy products with both spoilage and pathogenic bacteria (Austin and Bergeron, 1995; Marchand et al., 2012; Gopal et al., 2015), and are difficult to remove even with acceptable cleaning procedures (Bremer et al., 2006). For several years, numerous studies focused on optimizing cleaning-in-place (CIP) systems, by using different biofilm models

(Bénézech et al., 2002; Parkar et al., 2004; Faille et al., 2013; Kumari and Sarkar, 2014). General systems for the study of dairy biofilms often comprise complex settings that differ from laboratory to laboratory. This illustrates the need for techniques that rapidly and accurately characterize these biofilms and provide reproducible data with regard to their prevention and control. Biofilms that form at air-liquid interfaces are relevant models for the study of biofilm formation in aerobic sporeforming

bacteria such as *Bacillus cereus* (Wijman et al., 2007) and the thermophilic *Geobacillus* or *Anoxybacillus* (Zhao et al., 2013). Another simple model is the microorganism carrier-surface method previously described by Maris (1992) for testing the effectiveness of sanitizers. The formation of non-submerged biofilms on open surfaces is a practical method that was also used in other biofilm studies (Sommer et al., 1999; Leriche and Carpentier, 2000).

Microscopic methods are crucial for analysis of surface-associated communities. High-resolution imaging such as scanning electron microscopy (SEM), or confocal laser scanning microscopy (CLSM) proved to be powerful tools for biofilm structure deciphering, but they require extensive sample preparation (SEM) or image analysis (CLSM) and are not suitable for routine use. An easier microscopic approach is the use of environmental scanning electron microscopy (ESEM), which allows the observation of microbial biofilms in their native state without fixation, dehydration or metal coating (Little et al., 1991; Alhede et al., 2012). The main advantages of biofilm ESEM imaging are, indeed, the visualization of the highly hydrated organic matrix surrounding the bacteria, and minimal processing of samples.

In order to have, in a simple and rapid way, an insight into authentic biofilms that develop on dairy processing equipment surfaces, this study was carried out. A set of 13 strains of *B. cereus* and 10 strains of *Geobacillus* spp. of dairy origin were used to form biofilms on non-immersed stainless steel coupons, according to the method of Maris (1992). Non-submerged biofilms were further readily observed in ESEM. To evaluate the utility of this approach, non-submerged biofilms were compared to dairy biofilms *in situ*, inside milk processing lines.

MATERIALS AND METHODS

Bacterial strains culture and media

B. cereus were selected strains of genotypes that recurred for several years in a pasteurized milk processing line (Malek et al., 2013) whilst *Geobacillus* spp. are recent isolates from a monthly sampling performed in the same processing line (Table 1). Pipe-line surfaces were sampled with sterile swabs and dilution series made in TSE (0.1% trypton, 0.9% NaCl), plated on trypton-soy agar (TSA) medium (Sigma-Aldrich) and incubated at 55°C. Identification to *Geobacillus* spp. was performed according to thermophilic bacilli characteristics summarized in Burgess et al. (2010). Both *B. cereus* and *Geobacillus* spp. dairy isolates were characterized by high biofilm-forming potential on stainless steel coupons as revealed by plate count in agar media, respectively at 30 and 42°C (data not shown).

Preparation of stainless steel coupons and spore suspension

Stainless steel chips (AISI 304 L, 2 × 2 cm) were treated according to the protocol described by Peng et al. (2001). Spore suspensions of *B. cereus* and *Geobacillus* spp. were respectively obtained on Luria-Bertani agar (LB) and tryptic-soy-agar (TSA) media (Sigma-Aldrich), according to the method of Simmonds et al. (2003), and

prior to use, they were washed one time and suspended in saline (0.15 M) at pH 7.4.

Biofilm formation on non-submerged stainless steel coupons

Biofilms were performed on stainless steel chips, as previously described (Maris, 1992). Briefly, 100 µL of *B. cereus* or *Geobacillus* spp. spore suspensions (10^7 - 10^9 spores.mL^{-1}) were deposited onto clean and sterilized stainless steel coupons and allowed to adhere respectively at 30°C, and 45°C in a humidity-saturated atmosphere, for 3 h. Coupons were further washed with distilled water to remove weakly adhered cells, and then 100 µL of biofilm culture medium was poured over the adhesion area. Two culture media were used, the highly nutritious TSB medium (Sigma-Aldrich) and comparatively lesser nutritious nutrient broth Conda Pronadisa medium. Fouling coupons were incubated at appropriate temperatures for 20 to 24 h. In the case of the spore deficient strain *B. cereus* BC3 (Table 1), vegetative cells obtained from an overnight culture in LB broth, were harvested by centrifugation, washed with saline solution, and used to form biofilm on stainless steel coupon as described for spore suspensions.

Biofilm formation in co-culture

For biofilm formation in co-culture, spore suspensions (10^7 spores.mL^{-1}, v/v) of a strain of *B. cereus* (BC15, or BC9) and a strain of *Geobacillus* spp. (HP, or EP) were mixed in an eppendorf tube and then a drop of 100 µL was deposited onto stainless steel chips as described previously. The coupons were incubated at 45°C in a humidity-saturated atmosphere. This temperature which revealed suitable for biofilm formation by these *Geobacillus* spp. dairy isolates also suits for the mesophilic *B. cereus* goups III and IV previously described to grow between 15 and 45°C (Guinebretière et al., 2008).

Biofilm formation *in situ* inside milking pipes

Milk processing line surfaces from which *B. cereus* and *Geobacillus* spp. strains originated, were investigated for the formation of biofilms. The observation of biofilms *in-situ* requires the installation of tools or devices into processing lines. To achieve this in an easier manner, stainless steel coupons (1 × 2.5 cm) were pierced at their ends making either one or two holes of 3 mm diameter, cleaned and sterilized as described previously, and suspended, in several areas inside milking pipes, notably pre and post-pasteurization sections. After a week, the coupons were removed from pipes, 7 h after cleaning-in-place (CIP) procedures, that is, just before a new production run. The CIP regimen followed at the investigated dairy plant consists of alkaline wash (2% NaOH) for 10 min at 80°C and acid wash (1% HNO$_3$) for 5 min at 70°C.

Microscopy

All the above described biofilm carrying stainless steels coupons were gently rinsed with distilled water at the end of incubation time and examined in a 100 TM Hitachi environmental scanning electron microscope, at pressure in microscope chamber of 4 Torr.

RESULTS

Non-submerged 20 h old biofilms produced on stainless steel coupons by *B. cereus* or *Geobacillus* spp. dairy

Table 1. List and origin of dairy isolates studied.

Strain	Origin
B. cereus[a]	
BC1, BC5, BC9	Milk powder
BC2, BC3[b], BC4, BC7, BC13	Pre-pasteurization segment
BC15, BC16	Post-pasteurization segment
Geobacillus spp.	
HP, EP, MP, NP, PP, SP	Milk powder
H11, H17, H23	Equipment surfaces
LPM, LPP, LPN, LPR	Pasteurized milk*

[a]*B. cereus* dairy isolates were kindly characterized at the genotypic level at UMR 408 INRA Avignon, France in a previous work (Malek et al., 2013), and affiliated to the mesophilic *B. cereus* phylogenetic groups III and IV according to the classification of Guinebretière et al. (2008). [b]*B. cereus* strain BC3 is a spore deficient strain, which lost its ability to sporulate due to frequent culture transfers. The absence of spores was observed in contrast phase microscope for more than 5 days old cultures. *In the investigated dairy plant, pasteurized milk is obtained from reconstituted and processed milk powder.

isolates were elaborate compact or more extensive complex structures, well resolved in ESEM. A selection of the best replicated images of the biofilms produced by representative strains of *B. cereus* (BC2, BC4, BC15, BC1, BC5, BC16 and BC3) and representative strains of *Geobacillus* spp. (LPM, H11, HP, EP and H17) was chosen to be presented here. For both bacteria, ESEM images showed either intact mature biofilms or dispersion process. In *B. cereus*, dispersion occurred in nutrient broth, whereas TSB medium supported the development of substantial mature biofilms. In comparison, biofilm dispersal was observed in both media for *Geobacillus* spp. strains. It should be noted that this simple biofilm non-submerged model, produced wide structure diversity, and was efficient for analysis of biofilms at various developmental stages.

Dispersion from *B. cereus* biofilms

Figure 1 shows dispersion from luxurious biofilms which consisted of dense layers of rod-shaped cells interspersed with extracellular polymeric substances (EPS) material better visualized at higher resolution (Figure 1d). Dispersion occurred either from the outer surfaces of high colony diameter mushroom-like biofilms (Figure 1a) or through the formation of hollow central cavities characteristic of seeding dispersal (Figure 1c). Individual vegetative cells or small clusters of cells, rarely spores, were released from these biofilms and observed invading the substratum. At more advanced stages of dispersal process (Figure 1e and f), spores probably located in deeper areas within the biofilm, were released. The end of biofilm dispersal was imaged as cell-free EPS debris (Figure 1f).

Similarly, several matrix-rich biofilms were imaged in ESEM at the dispersion stage (Figure 2). The mosaic biofilm shown in Figure 2a was characterized by irregular matrix blocks stacked on each other, delimiting large voids from which spores were released. Spores also dispersed from artistically shaped biofilm matrix (Figure 2c to d) and beside spores, vegetative cells were released from the pillar-like biofilm (Figure 2c), as shown following biofilm breaking (Figure 2d). In this biofilm, it appears that both vegetative cells and spores were rather embedded within the amorphous dark grey EPS-material, visualized under light grey pillar-like structures. This is clearly suggested in Figure 2d, where void areas resulting from the dissolution of dark grey material are observed, without obvious alteration of the pillar-like structures, at this stage. These results clearly indicate that spores were more present in matrix-rich *B. cereus* biofilms, in comparison to the above described luxurious biofilms. In addition the biofilm formed by the spore deficient *B. cereus* strain BC3, consisted of dense layers of rod-shaped cells, organized in a marked three-dimensional architecture without obvious spores and with nearly no visible EPS-matrix (Figure 3).

Mushroom-like and heterogeneous structures of *B. cereus* biofilms

In comparison to nutrient broth, *B. cereus* developed densely packed complex biofilms in the highly nutritious TSB medium. Figure 4b shows a complex biofilm characterized by a heterogeneous mushroom-like structure, penetrated by interstitials voids and large deep water channels. ESEM images clearly depicted this remarkable network of well-defined channels which either
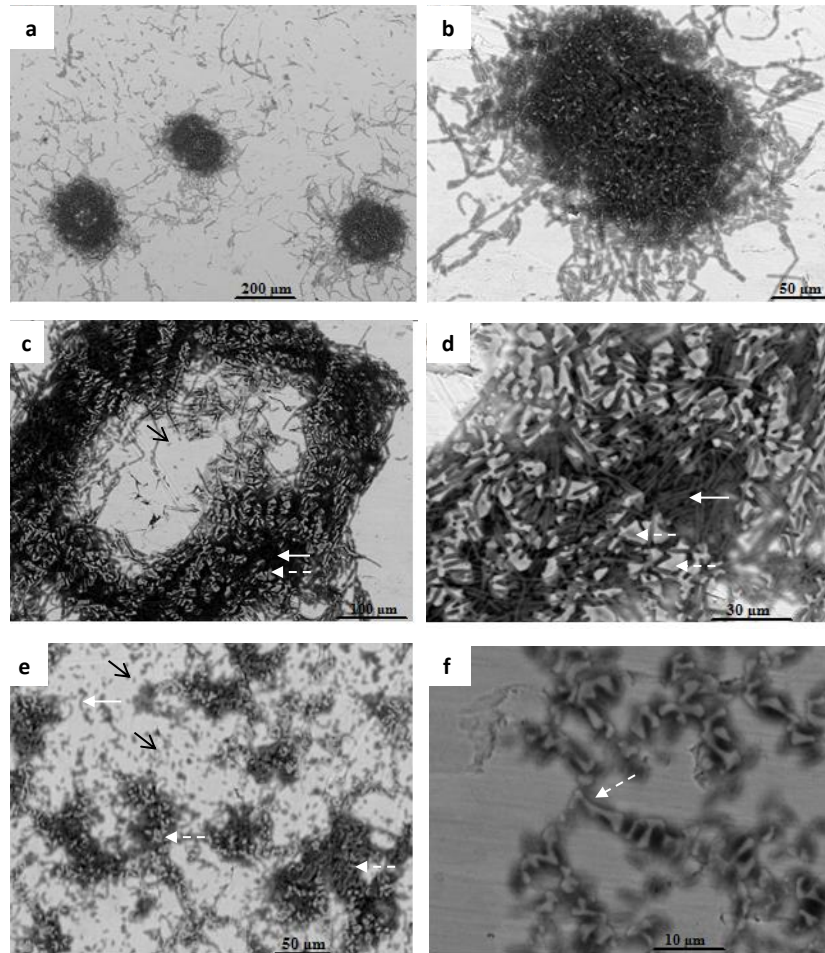
Figure 1. Biofilm dispersal in *B. cereus* luxurious biofilms. (a-d) Seeding dispersal from high biofilm colony diameters formed at various sites of the same stainless steel coupon by *B. cereus* strain BC2. (b) Central biofilm in (a) at higher magnification. (e and f) Selected areas of more advanced seeding dispersal stages of *B. cereus* strain BC4. White dashed arrows indicate portions of the biofilm matrix. White arrows indicate rod-shaped cells and black arrows spores. Biofilms were formed in nutrient broth.

extended from the top to the bottom of the biofilm (Figure 4b) or occurred at its surface (Figure 4c). It was also easy to view the marked widening and profound depth of these galleries. We can also see other heterogeneous topographical characteristics of these complex mature biofilms where cells were entirely encased in the EPS matrix. In contrast, Figure 4a showed another mushroom-like biofilm characterized by a compact porous structure, formed in comparatively more limited nutritious medium (Nutrient broth).

Dispersion from *Geobacillus* spp. biofilm

Geobacillus spp. strains developed densely packed mature biofilms undergoing an original dispersing style as well as extensive spread structures. Figure 5a showed large heterogeneous biofilms developed in crevices. Central parts of this biofilm were surrounded with large pieces of homogeneous EPS-matrix. Various geometrically shaped structures, better visualized at higher resolution (Figure 5c and d), were scattered at the upper surface of the biofilm. In this heterogeneous and closed structure, vegetative rod-shaped cells were released through small holes. At the upper surface of another part of this biofilms we can also see the EPS-matrix elevated by some hidden items in a piercing process (Figure 5b). This procedure is clearly understood, in Figure 5c and d. The emerging materials are slightly sharp tools, which pierced the EPS matrix, enabling cells to escape, from the biofilm. Figure 6 shows more conventional dispersion styles resulting from matrix degrading or through the formation of central voids in extensive structures (Figure 7) as well as detachment of
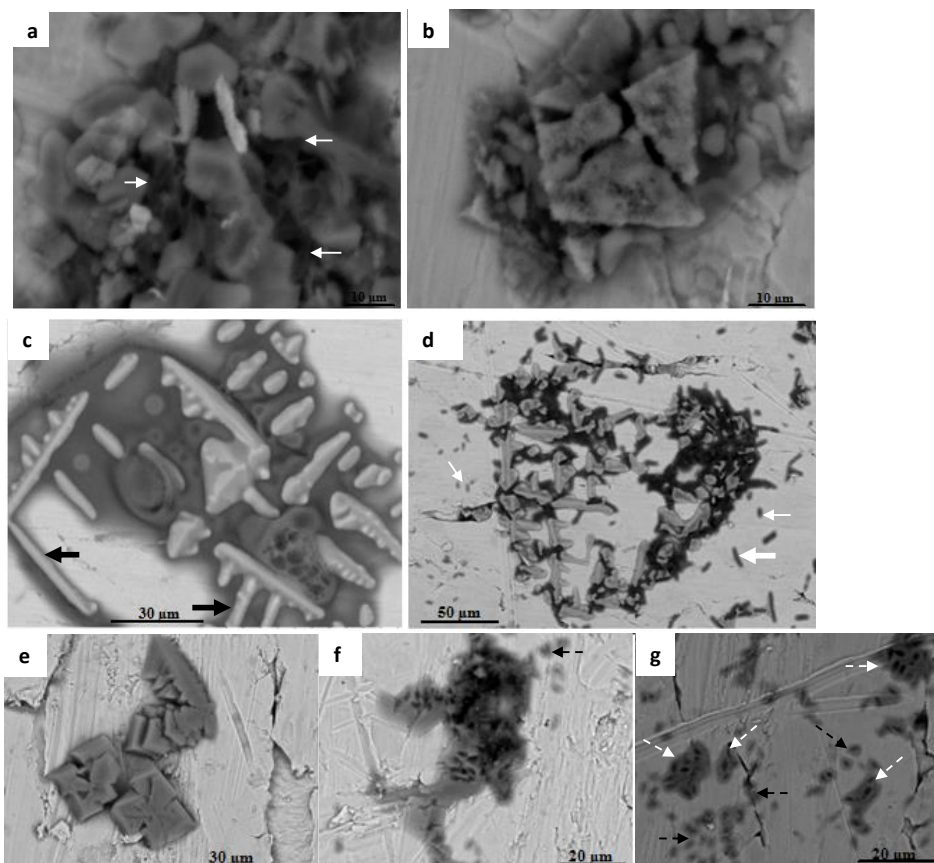
Figure 2. Biofilm dispersal in matrix-rich *B. cereus* biofilms. (a) Spore dispersion from a heterogeneous mosaic biofilm of *B. cereus* strain BC15. (b) Selected area of the biofilm shown in (a). (c) pillar-like biofilm structure of *B. cereus* strain BC1. (d) Dispersion from a pillar-like biofilm. (e) An artistically shaped homogeneous biofilm of *B. cereus* strain BC5. (f and g) Dispersion from homogeneous biofilms. White arrows indicate planktonic spores and white arrows in bold vegetative cells. Black dashed arrows present EPS-surrounded spores and white dashed arrows detachment of portions of the biofilm (sloughing). Black arrows in bold indicate pillar-like structures. Biofilms were formed in nutrient broth.

small portions from a heterogeneous octopus-like biofilm (Figure 8).

Non-submerged mixed-species biofilm

Mixed-species biofilms formed by a mixture of *B. cereus* strain BC15 and *Geobacillus* spp. strain HP were heterogeneous mosaics (Figure 9), invoking the structure of the biofilm formed individually by *B. cereus* strain BC15 (Figure 2b). It seems that, in co-culture at 42°C, *B. cereus* was best able to grow in biofilm than *Geobacillus* spp.

Biofilms *in situ* inside milking pipes

The biofilms formed on suspended stainless steel coupons installed during a week, in lines carrying

pasteurized milk are extensive structures characterized by amorphous matrix, and high surface coverage (Figure 10). In post-pasteurization segments these surface-associated structures seem to be more influenced by the flux effect than in pre-pasteurization segments. Younger biofilms developed in both segments probably between two production runs (Figure 10b and d).

Another highly distinct biofilm structure formed on stainless steel coupons installed at another site of the pre-pasteurization section is shown in Figure 11b and c. Straight or curved rod-shaped matrix characterized these biofilms, which strikingly resemble non-submerged *in vitro* biofilms formed by strains of *Geobacillus* spp. in nutrient broth (Figure 11a). It seems that all these structures, probably formed under unsuitable conditions may be devoid of living cells. The similarities recorded for biofilms formed *in situ* and those formed *in vitro*, highlights the usefulness of the non-submerged assay for the study of biofilms.
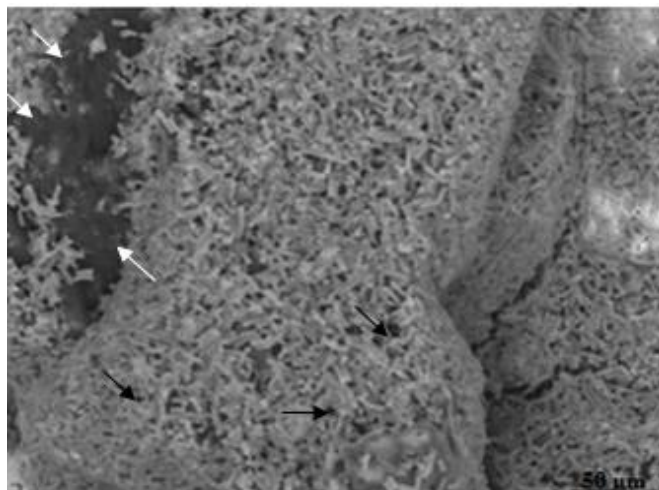
Figure 3. The biofilm formed by the spore deficient *B. cereus* strain BC3. ESEM image shows dense layers of rod-shaped cells organized in a marked three-dimensional structure. White arrows indicate a portion of the EPS-matrix used as a scaffold for the 3D architecture, and black arrows deep small holes.
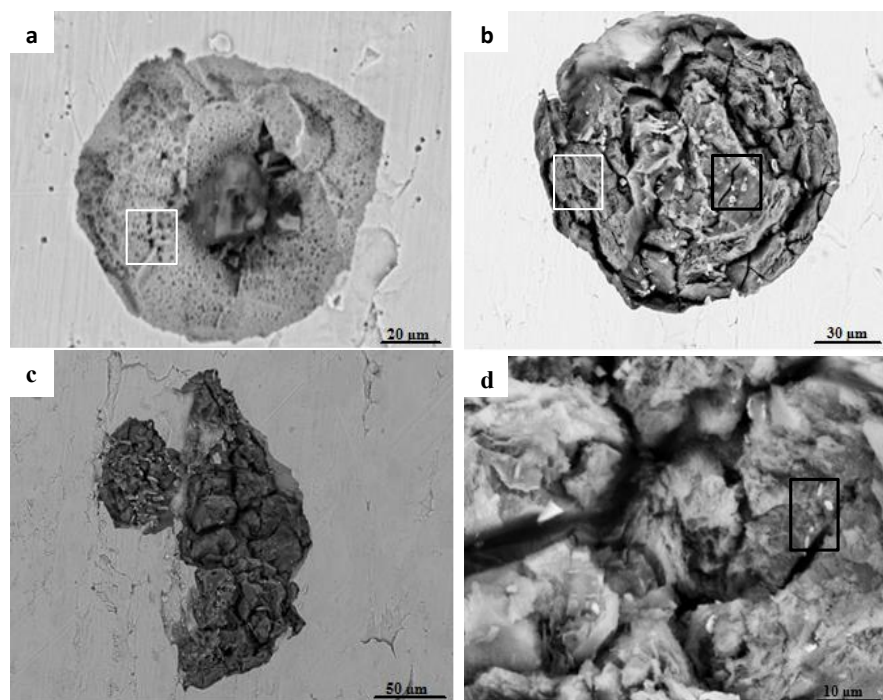
Figure 4. Mushroom-like structure and channeled networks of *B. cereus* biofilms (strain BC16). ESEM pictures show porous or channeled mushroom-like biofilms respectively formed in nutrient broth (a) and TSB (b). (c) Heterogeneous channeled biofilms spread in crevices of the same coupon as (b). (d) Central area of (c) at higher magnification. Black frameworks indicate small white structures scattered at the upper surface of biofilms probably used to create small holes (white frameworks), for cell dispersal.

DISCUSSION

This study proposes a method that relies on the use of environmental scanning electron microscopy combined with a simple cultivation, technique which has proved to be compatible with high resolution imaging, for the study
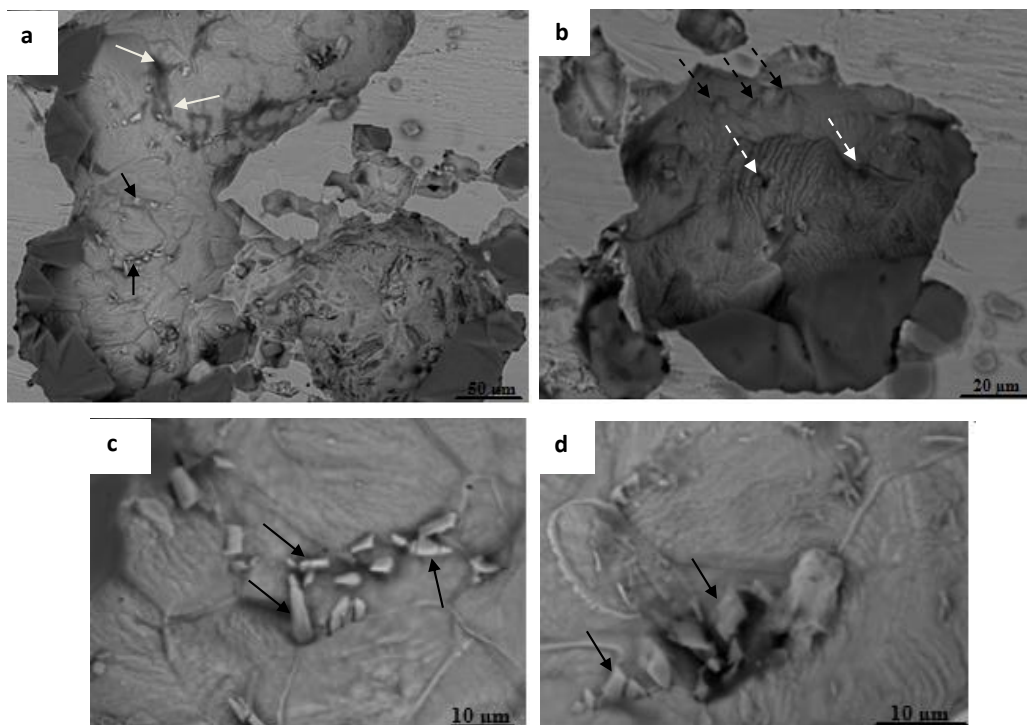
Figure 5. An original dispersal style in *Geobacillus* spp. strain LPM biofilms. (a and b) Complex compact biofilms formed in large crevices of the same stainless steel coupon. (c and d) areas of (a) at higher magnification. White arrows showed vegetative cells released from the biofilm through small holes in the EPS-matrix. Other small holes are obvious at different points of the upper surface of these biofilms (white dashed arrows). Black arrows show various well-defined sharped structures emerging from the matrix or still piercing it (black dashed arrows).
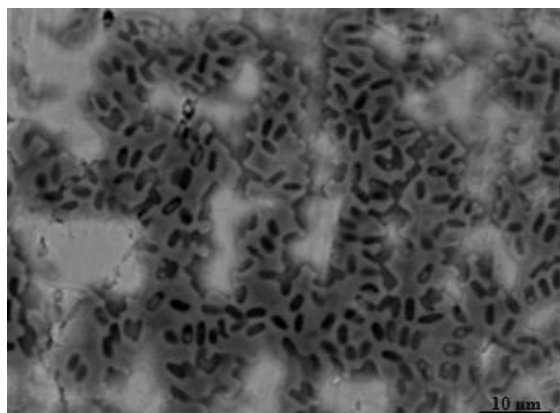
Figure 6. Dispersal from homogeneous biofilms formed in nutrient broth by *Geobacillus* spp. strain H11**.** Images show deep cell fingerprints devoid of cells.

of biofilm structures. ESEM provided excellent resolution of dairy biofilms formed *in vitro* and *in situ* inside industrial milking pipes, and revealed structures not previously described for *B. cereus* or *Geobacillus* spp.

biofilms. It is noticeable that ESEM images of *B. cereus* non-submerged biofilms are, at some extent, comparable to those obtained in SEM or CLSM for immersed biofilms formed by other bacteria (Gulot et al., 2002; Parsek and Fuqua, 2004), highlighting the usefulness of this model for the study of biofilms.

Results showed that both *B. cereus* and *Geobacillus* spp. biofilms underwent rapid dispersion within 20 h or lesser, in comparison with previous work (Wijman et al., 2007). This indicates that this biofilm non-submerged model supported a luxurious growth resulting in rapid dispersal. In addition, dispersion occurred in the way called seeding dispersal or central hollowing, previously described for *Pseudomonas aeruginosa* biofilms cultured in flowing systems (Boles et al., 2005; Kirov et al., 2007). Indeed, non-submerged biofilms developed under static conditions were substantial structures with high colony diameters of > 80 µm, previously reported as a threshold required for hollow cavity formation to occur in *P. aeruginosa* biofilms (Purevdorj-Gage et al., 2005). The structural similarities recorded for biofilms formed in batch system and biofilms in dynamic systems increases the score of the non-submerged assay as an efficient biofilm cultivation method. Interestingly, central hollowing was also found in complex biofilms developed in dairy
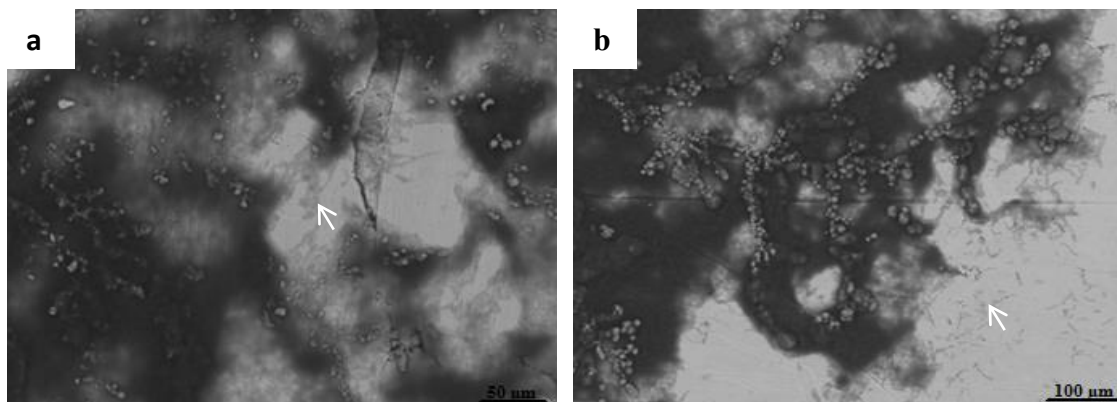
Figure 7. Central hollowing in extensive biofilms of *Geobacillus* spp. strain HP. ESEM images show EPS-enmeshed cells containing several internal cavities characteristic of seeding dispersal and crossed by complex networks of secreted EPS formations.
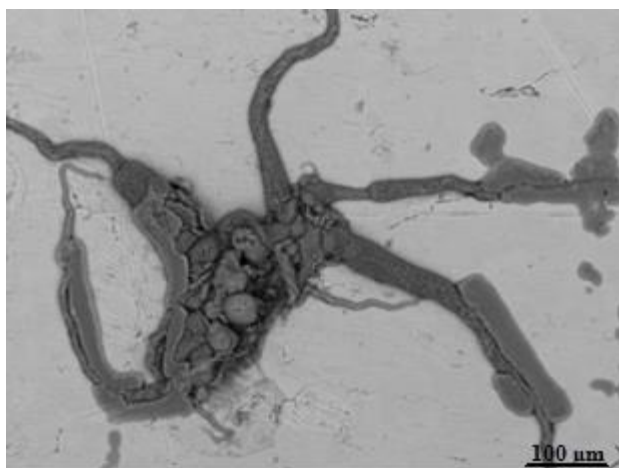
Figure 8. Structural diversity of *Geobacillus* spp. biofilms. An overview at low magnification of a heterogeneous octopus-like biofilm formed in TSB by *Geobacillus* spp. strain EP.
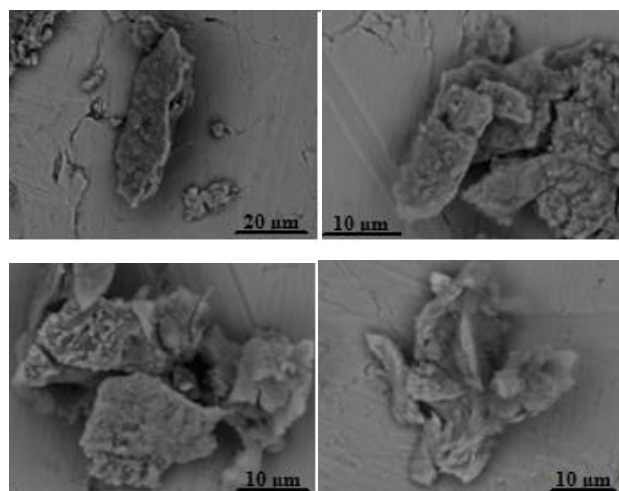
Figure 9. Mosaic structures of non-submerged mixed-species biofilms formed by a mixture of *B. cereus* strain BC15 and *Geobacillus* spp strain HP.

processing lines. Other conventional dispersal processes observed in non-submerged biofilms, included sloughing, erosion and cell release from mosaic or uniform matrix biofilms.

Non-submerged biofilms formed by strains of *Geobacillus* spp. displayed an original dispersion style not previously described. Cells were released through small holes performed in the matrix using sharped tools. This strategy to leave the biofilms was also observed in *B. cereus* compact mushroom-like biofilm. Unlike recognized dispersing mechanisms, this new strategy to escape from the biofilm appears not to rely upon biochemical matrix degrading, but, on a physical process which consisted of piercing the matrix surface using well-defined geometrical sharped structures most likely of crystalline nature. Crystal structures in biofilms have mainly been ascribed to mineral formations in specific biofilms characterized by high rates of minerals (Holling et al., 2014). Such high content in minerals has neither been reported for *B. cereus* matrix biofilms, mainly composed of polysaccharides, proteins and eDNA (Vilain et al., 2009), nor for thermophilic bacilli biofilms. Thereby, the observed piercing tools should rather be organic formations mainly polysaccharides or/and proteins.

Detachment or dispersion is the final stage of biofilm development and an essential stage of the biofilm cycle life. This process has a crucial meaning with regard to cross contamination and disease transmission. That is why, in recent years, dispersion was considered as an interesting target for biofilm prevention and control strategies, in industrial and clinic settings (Kaplan, 2010; McDougald et al., 2011, Rabin et al., 2015). Interestingly, non-submerged biofilms combined to ESEM appears as
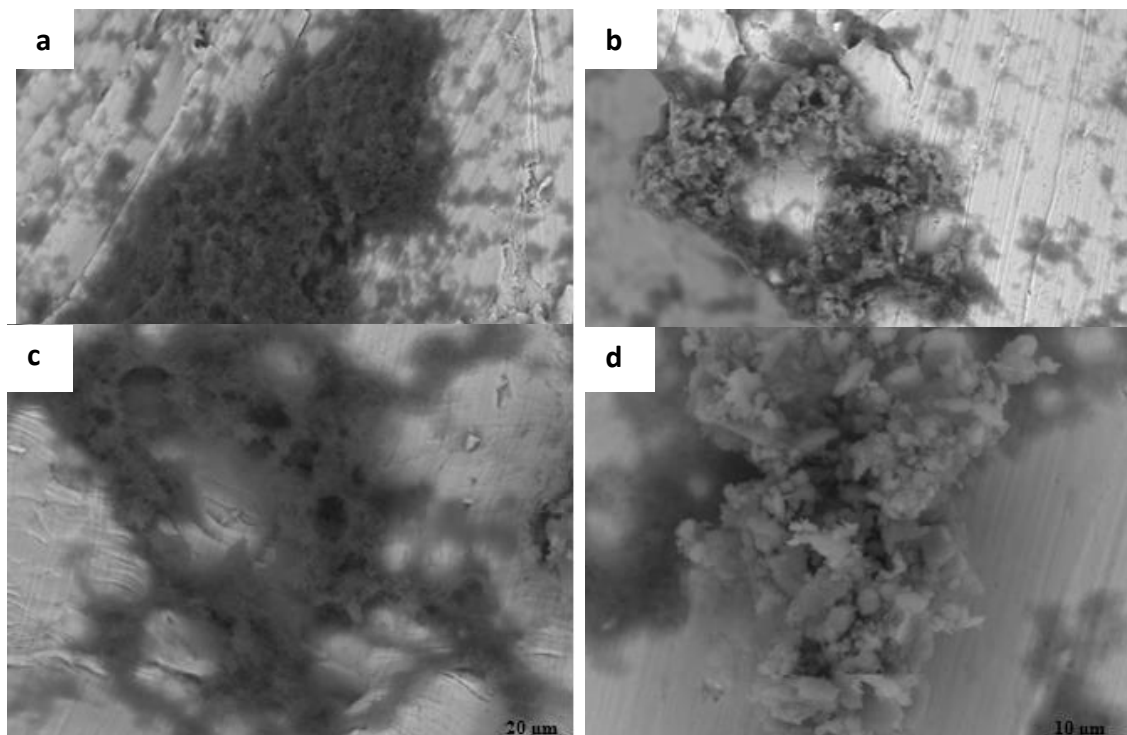
Figure 10. Dairy biofilms *in situ* on stainless steel coupons introduced inside milking pipes. (a) An old mature biofilm and (b) heterogeneous biofilm displaying dispersing areas characterized by central hollowing, both formed at different points on the same stainless steel coupon in pre-pasteurization segment. (c) Sheared surface-associated structures and (d) Young heterogeneous biofilm, both formed in post-pasteurization segment on the same stainless steal coupon.
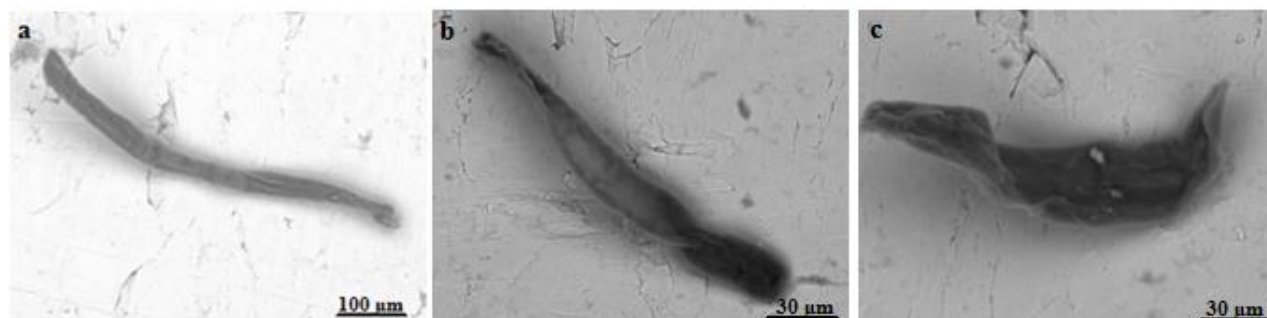
Figure 11. Rod-shaped structures of biofilms *in vitro* and *in situ*. (a) Non-submerged biofilm formed by *Geobacillus* spp. strain H17 in nutrient broth. (b and c) Straight and curved rod-shaped matrix of biofilms *in situ* inside pre-pasteurization pipes.

an efficient model for analysis of biofilm dispersion and should provide additional data and measurements, since samples examined in ESEM can be used with a range of downstream methods directly after viewing (Bergmans et al., 2005). This will permit in-depth investigations of biofilm dispersal notably the identification of the piercing tools.

Major differences in biofilm structures were not only strain dependent but also related to environment factors namely culture media and adhesion sites on the stainless steel coupons. The non-submerged assay successfully illustrated this wide structural diversity in biofilms formed by sporeforming bacteria, and produced elaborate three-dimensional or more extensive structures often characterized by complex networks of pores or water channels. ESEM pictures confirmed recent data concerning biofilm formation in mesophilic and thermophilic sporeforming bacteria (Gopal et al., 2015; Majed et al., 2016). *Bacillus* biofilms were previously shown to contain both spores and vegetative populations (Lindsay et al., 2006; Pagedar

and Singh, 2012). In addition, sporulation and biofilm formation have been shown to occur simultaneously in the thermophilic bacilli *Anoxybacillus flavithermus* (Burgess et al., 2010). In the current study, spores of *B. cereus* appeared more present in matrix-rich biofilms than in luxurious biofilms. Furthermore, in the biofilm formed by the spore deficient *B. cereus* strain BC3 (Figure 3), the amount of EPS is obviously lesser than in the obtained spore-induced biofilms. In good agreement, Simões et al. (2007) found small amount of EPS in the luxurious biofilm formed by *B. cereus*. Interestingly, these findings confirmed data concerning *B. cereus* biofilms developed in dynamic system, and provide additional evidence for the efficiency of the combination of non-submerged assay to ESEM as a model for biofilm analysis.

At the morphological level, ESEM imaging showed that, non-submerged biofilms often displayed homogeneous matrix, previously reported to be less stable compared to their heterogeneous counterparts (Anand et al., 2014), enhancing the risk of cell dissemination into processing environments. These findings are interesting cues for the dairy industry.

In good agreement with previous work (Austin and Bergeron, 1995), dairy biofilms formed inside milk processing lines, were mostly extensive structures characterized by amorphous matrix. The biofilm developed in pre-pasteurization sections, should result from a robust bacterial growth due to heavy contamination of milking pipe surfaces. Bacterial contamination of the process equipment in this dairy plant was found to occur at high levels (up to 10^7 cfu/cm^2) (unpublished data) and such cell density was reported to result in biofilms structures consisting of several layers (Gibson et al., 1999).

Therefore, dairy biofilms are characterized by the predominance of single species of bacteria, due to selective pressure from surrounding environments (Flint et al., 1997). In the investigated dairy plant, mesophilic and thermophilic sporeformers were predominant groups of constitutive bacteria of the biofilms developed on milk processing line surfaces. Strikingly, certain biofilms *in situ* displayed similar structures to those developed *in vitro* by *B. cereus* or *Geobacillus* spp. and should be attributed to the development of one or the other but not to both organisms. Indeed, the non-submerged mixed-species biofilms consisted of widely distinct structures imaged as heterogeneous mosaic biofilms (Figure 9).

Overall, the biofilm system used *in situ* provided valuable data concerning the hygienic statute of the investigated dairy plant indicating the failure of conventional cleaning procedures to remove spores and biofilms. The development of young biofilms on post-pasteurization segments, after CIP procedures as well as detachment of small portions from older biofilms, highlights high cross contamination risk. In conclusion, the microorganism carrier-surface method combined to

ESEM revealed a relevant model for the study of dairy biofilms. The cultivation of biofilms on non-immersed surfaces produced a diversity of *B. cereus* and *Geobacillus* spp. biofilm architectures and specific dispersion features. This model is rapid, easy to control and suitable for routine use. Moreover, considering its efficiency for testing the effectiveness of sanitation agents, this model is of major concern to the dairy industry. Non-submerged biofilms produced under static conditions were also comparable to those developed in dynamic systems as well as to biofilms *in situ* in dairy processing lines. This clearly suggests that data of the colonization of open surfaces should be extrapolated to closed piping systems, and overall, highlights the relevance of this approach for the study of biofilms.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Cytokinins production by fluorescent *Pseudomonas* isolated from rhizospheric soils of *Malus* and *Pyrus*

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Information on microbial production of cytokinins and their effect on plant growth are limited. Therefore, the objective of the present study was to investigate cytokinins production by *Pseudomonas* sp., a major component of rhizobacteria with multiform and diverse activities, which enhance plant growth by synthesizing various secondary metabolites. In the present investigations, thirty *Pseudomonas* isolates were isolated from the rhizosphere of *Pyrus* and *Malus* and were screened for cytokinins production (50 to 210 µg/ml). Four strains *viz* PN-4-SAN, PN-10-SAN, AN-2-NAG and AN-4-NAG were selected on the basis of their higher cytokinins production. The maximum cytokinins production was observed at 72h incubation period in nutrient broth at pH 7.0 under shaken condition at 28°C. Cytokinins were extracted, purified and evaluated by thin layer chromatography and specific bioassay method.

Key words: Cytokinins, plant growth promoting rhizobacteria (PGPR), *Pseudomonas* sp., thin layer chromatography, radish cotyledon bioassay.

INTRODUCTION

Fluorescent *Pseudomonas* species have emerged as largest and potentially most promising group of plant growth promoting rhizobacteria (PGPR). Their potential to synthesize different secondary metabolites like phytohormones with diverse and multiform activities is an important function to improve plant growth and health (Kloepper et al. 1988). Phytostimulatory effect of PGPR may be initiated by several ways but the production of phytohormone such as cytokinins and facilitating resource acquisition by such bacteria is direct mechanism of plant growth promotion (Garcia de Salamone et al., 2001; Glick, 2012; Ramadan et al., 2016) while indirect mechanism of action may involve antibiotic production, depletion of iron from the rhizosphere, synthesis of antifungal metabolites, production of fungal cell wall lysing enzymes, competition for sites on roots and induced systemic resistance (Glick, 2012). Phytohormone, cytokinins can be produced by many bacterial genera

and its occurrence was observed in many terrestrial and rhizospheric soil (Kampert and Strzelcyk, 1984; Arkhipova et al., 2007; Hussain and Hasnain, 2009) which suggested that bacterial cytokinins may affect the growth and development of plants. Recent studies confirmed that, a number of bacterial species mostly associated with the plant rhizosphere, are found to be associated with cytokinins production including *Flavobacterium*, *Acinetobacter*, *Bacillus*, *Arthrobacter*, *Aerobacter*, *Azospirillum* and *Pseudomonas* sp. (Maruyama et al., 1986; Perig et al., 2007; Arkhipova et al., 2007; Ortiz-Castro et al., 2008). Cytokinins have key regulatory roles in plant growth and development. They promote seed germination, *de novo* bud formation, release of buds from apical dominance, stimulation of leaf expansion and of reproductive development besides on, retardation of senescence (Mok, 1994). Some of these effects have been observed in cucumber cotyledon when inoculated with *Pseudomonas aeruginosa* E2 (Hussain and Husnain, 2009).

Cytokinins influence cell division activity in embryonic as well as mature plants by altering the size and activity of meristems (Werner et al., 2001). Yang et al. (2002) showed that the rate of endosperm cell division is closely associated with cytokinin level in endosperm. They also reported that exogenous kinetin significantly increase the number of endosperm cells and grain weight. Morris and Powell (1987) first reported on genes, specifying cytokinins biosynthesis from phytopathogenic bacteria notably *Agrobacterium tumefaciens* (tmr, tzs) and *Pseudomonas syringae* pv, *savastanoi* (ptz). Genes encode prenyl transferase is responsible for cytokinins biosynthesis. High levels of cytokinins were found toincrease the resistance of plants to some viral pathogens and herbivores (Ballare, 2011).

Großkinsky et al. (2016) identified the ability of *Pseudomonas fluorescens* G20-18 to efficiently control *P. syringae* infection in *Arabidopsis* and cytokinin production which was identified as a key determinant for this biocontrol effect on the hemibiotrophic bacterial pathogen, allowing maintenance of tissue integrity and ultimately biomass yield. Naseem and Dandekar (2012) also reported that cytokinin confers protection to *Arabidopsis* against infection with *Pseudomonas syringae* pv. *tomato* (*Pto*).

However, to date little information exists on the diversity of plant growth promoting rhizobacteria associated with apple and pear trees. Moreover, in apple and pear growing region of Himachal Pradesh, cytokinins producing *Pseudomonas* population has never been estimated.

In present work, plant growth regulators producing *Pseudomonas* sp. from rhizospheric soil of apple and pear trees were isolated and characterized for production of one of the important plant growth regulators i.e. cytokinins.

MATERIALS AND METHODS

Isolation of fluorescent *Pseudomonas* species from rhizosphere of apple and pear trees

Soil sampling

Soil samples were collected from rhizosphere of ten years old orchards of *Pyrus* (pear) from Sanyardi (31°41'42.1" N, 76°55'56.7" E) and *Malus* (apple) from Nagwain (31°48'57.9" N, 77°10'46.1" E) regions of Mandi district (Himachal Pradesh), India during autumn season (25°C). The pH of rhizospheric soil of pear and apple was found to be 7.3 and 7.5, respectively and electrical conductivity is 195 and 210 µS/cm, respectively. Four samples were taken around few to 10 cm apart from roots and 30 to 45 cm deep from five different plants and mixed to make it one composite sample.

Isolation and Identification

Total bacterial count viz- a-viz fluorescent *Pseudomonas* sp. was isolated on Nutrient agar and King's B media (Hi-media), respectively from soil samples. The proposed isolates were characterized on the basis of morphological, biochemical and physiological analyses as prescribed in Bergey's manual of systematic bacteriology by Kreig and Holf (1984).

Screening of isolates for cytokinins production

For production of cytokinins, *Pseudomonas* strains (Inoculum size 10^8 cfu/ml) were grown in nutrient broth for 72h at 28 ± 2°C under shaken conditions. Supernatant was prepared by centrifugation of cultures at 10,000 rpm for 20 min and was stored in deep fridge or at 4°C. Radish cotyledons expansion test was employed (Letham, 1971) for assay of cytokinins like, substances; the radish seeds (*Raphanus sativus* L. cultivars Japanese white) which were germinated in total darkness for 48 h at 28°C. After removing the seed coat, smaller cotyledons were transferred to sterilized Petri dishes containing the test solution, water (as blank) and standard solution (kinetin) on filter paper strips. Twelve cotyledons were placed in each Petri dish and kept at 25°C under fluorescent light for 3 days. Then cotyledon on filter paper strips in Petri dish were blotted, dried and weighed. The bioassay response (final weight-initial weight) was expressed as an increase in weight. Concentration of cytokinins present in the extract was calculated by preparing standard curve using kinetin as standard (100 to 1000 µg/ml).

Effect of culture conditions on production of cytokinins by Pseudomonas sp.

The test organisms (10^7 cfu) were inoculated in five different media; succinate media, king's media, nutrient media, peptone water and trypticase soyabroth. Flasks were incubated at 28°C for 24, 48, 72 h under shaker at 90 rpm. After this step, supernatant were picked up by centrifugation in multifuge XIR (Thermoscientific Germany) at 10,000 rpm for 30 min at 4°C and were further used for estimation of cytokinins.

Extraction and separation of cytokinins

Cytokinins like substance were extracted by the method of Tien et
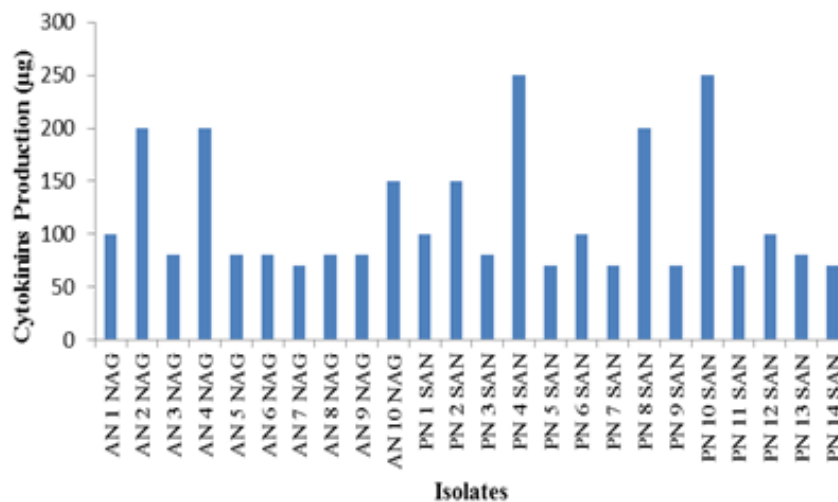
Figure 1. Cytokinins production by fluorescent *Pseudomonas* strains isolated from Apple (AN-NAG) and Pear (PN-SAN).

al. (1979) with slight modifications. Acidified supernatant extracted with di-ethyl ether and partitioned with n- butanol. Residue was dissolved in 0.1 N HCl (10 ml) and passed through Dowex-50 column chromatography and eluted with sodium hydroxide. Extracted and concentrated fraction was diluted in distilled water. Collected fraction was separated by thin layer chromatography by spotted (100 µl) on silica gel-G plates and developed in n-butanol: ammonium hydroxide (I N): water (7:1:2). The plates were examined under U.V light (254 nm) and observed for the fluorescent spots.

$$\text{Rf value calculated as} = \frac{\text{Distance run by the compound}}{\text{Distance run by the solvent front}}$$

Evaluation of cytokinins by Radish cotyledons expansion test

The partially purified cytokinins were evaluated by radish cotyledon expansion test. Radish seeds were surface sterilized with 0.1% $HgCl_2$ for 3 min and rinsed five times with sterile distilled water and germinated in total darkness for 48 h at 28°C. After removing the seed coat smaller cotyledons were transferred to sterilize Petri dishes containing 7ml test solution (extracted sample), water (control) and standard solution (Kinetin) 100 to 1000 µg on filter paper strips. Twelve cotyledons were placed in each Petri dish and were incubated at 25°C under fluorescent light for three days. Each treatment was replicated three times. The cotyledons were filtered and weighed. The bioassay doses response curve drawn (initial weight to final weight) was expressed as increase in weight against concentration of cytokinins present in the extract and calculated by preparing standard doses responses curve by using kinetin as standard (Letham, 1971).

Statistical analysis

The data obtained was subjected to analysis of variance technique using completely randomized design (CRD).

RESULTS

The rhizospheric soil samples were evaluated for the status of total bacterial population viz a viz *Pseudomans* sp. Total bacterial count of rhizospheric soil of apple and pear was 6.1 and 6.3 log cfu/g of soil, respectively. A total of seventeen *Pseudomonas* isolates were obtained from rhizosphere soil of pear plant and thirteen were obtained from rhizosphere soil of apple plant up to its maturity level. On the basis of morphology and biochemical analyses, these isolates were identified as Gram negative, rod shaped bacteria which produce fluorescence under ultra violet radiations and showed positive test for catalase and oxidase.

In our studies it was found that all *Pseudomonas* isolates produced cytokinins in the range of 70 to 250µg/ml (Figure 1). Four strains *viz* PN-4-SAN, PN-10-SAN, AN-2-NAG and AN-4-NAG were selected on the basis of their higher cytokinins production. The efficient isolates produced cytokinin like substances in the stationary growth phase i.e. at 72h of incubation period at 28°C in nutrient broth (Table 1).

The homogeneity of the partially purified cytokinins along with kinetin as standard was checked by thin layer chromatography. Cytokinins gave the maximum Rf value of 0.75. Brown spots corresponding to, cytokinins or cytokinins like substances were visible when sprayed with reagent (Table 2 and Figure 2). As the result showed partially purified cytokinins extracted samples from *Pseudomonas* sp. PN-4-SAN, PN-10-SAN, AN-2-NAG and AN-4-NAG have increased the weight of radish cotyledons by 0.02 to 0.2 cm respectively that correspondent to 100 to 200 µg/ml as calculated from the dosage response curve of kinetin (Table 3 and Figure 3).

Table 1. Effect of media on the production of cytokinins (µg/ml) by fluorescent *Pseudomonas* at different incubation period.

Media	PN-4-SAN				PN-10-SAN				AN-2- NAG				AN-4-NAG			
	24	48	72	Mean	24	48	72	Mean	24	48	72	Mean	24	48	72	Mean
Succinate media	150	200	280	210	70	150	200	140	100	150	200	150	100	150	200	150
King's media	100	150	200	150	100	150	280	176.7	150	200	280	210	150	200	280	210
Nutrient broth	150	200	280	210	200	250	280	243.3	200	250	280	243.3	200	250	280	243.3
Peptone water	100	150	200	150	70	150	200	140	100	150	200	150	100	150	200	150
Trypticase soy broth	140	170	200	170	70	150	50	106.7	70	150	220	140	70	150	220	140
Mean	128.9	174	232		102	160	222.6		124	180	236		124	180	236	

Table 2. Thin layer chromatographic analysis on Silica gel-G of partially purified bacterial cytokinins from *Pseudomonas* sp.

Isolates	Solvent system	Spraying reagent	Color of spots	Rf value
PN-4-SAN	nbutanol:1N NH$_4$OH:Water (7:1:2)	Iodine	Brown	0.73
N-10-SAN	-do-	-do-	Brown	0.75
AN-2-NAG	-do-	-do-	Brown	0.75
AN-4-NAG	-do-	-do-	Brown	0.72
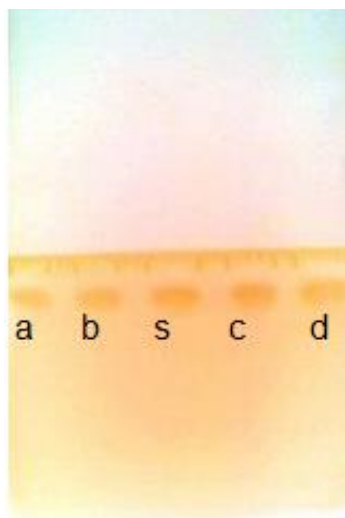
Figure 2. Thin layer chromatographic pattern on silica gel-G of partially purified cytokinins of *Pseudomonas* sp. PN-4-SAN (a), PN-10-SAN (b), AN-2-NAG (c), AN-4-NAG (d) and kinetin (s) using n-butanol: 1N NH4OH: Water (7:1:2) and developed in iodine chamber.

DISCUSSION

Cytokinins are important class of phytohormones which are adenine derivatives. Phytostimulatory effects of PGPR may be initiated by several ways but cytokinin production by such bacteria is the direct mechanism to improve plant growth (Ortiz-Castro et al., 2008; Remans et al., 2008). Cytokinins producing bacteria i.e. *Pseudomonas*, *Rhizobium*, *Bacillus* and *Agrobacterium* have been isolated from various crop plants including maize, wheat, tomato, sugarcane, mustard, carrot and sunflower (Hussain and Hasnain, 2009). However, in our studies, *Pseudomonas* strains producing cytokinins were obtained from rhizospheric soil of apple and pear. Significant difference was observed in total bacterial and *Pseudomonas* communities of apple and pear orchards.

In this study, cotyledon bioassay was used to identify efficient *Pseudomonas* strains producing cytokinins like substance, as it was already found to be convenient and sensitive by providing a more linear response (Dumbroff and Brown 1976). Based on the data presented, the cytokinins yield was maximized (280 µg/ml) at 72 h of incubation at pH7 under shaken conditions at 28°C. It might be due to the reason that cultural conditions play an important role in cellular growth and biological activities of microorganisms as stated by Bentley and Keil (1962). Hussain and Hasnain (2009) reported cytokinin as equivalent to 1091.9 ng ml^{-1} in the late stationary phase culture of *Bacillus licheniformis*. In another study, Taller and Wong (1989) determined cytokinins as equivalent to 0.75 µg of kinetin per litre in *Azotobacter vinelandii* culture medium while Karnwal and Kaushik (2011) reported 5.5 and 2.9 pmol/ml of cytokinins per litre for *P. fluorescens* and *Pseudomonas aeruginosa*,

Table 3. Effect of partially purified cytokinins of fluorescent *Pseudomonas* on the weight of radish cotyledon seeds.

Partially purified cytokinins	Increased weight (% units)	Cytokinins*(µg/ml)
PN-4-SAN	0.04	150
PN-10-SAN	0.04	150
AN-2-NAG	0.02	70
AN-4-NAG	0.03	100

*Cytokinins production expressed in terms of kinetin (µg/ml) as calibrated from the dose response curve (100-1000µg/ml) Each value is the mean of triplicate.
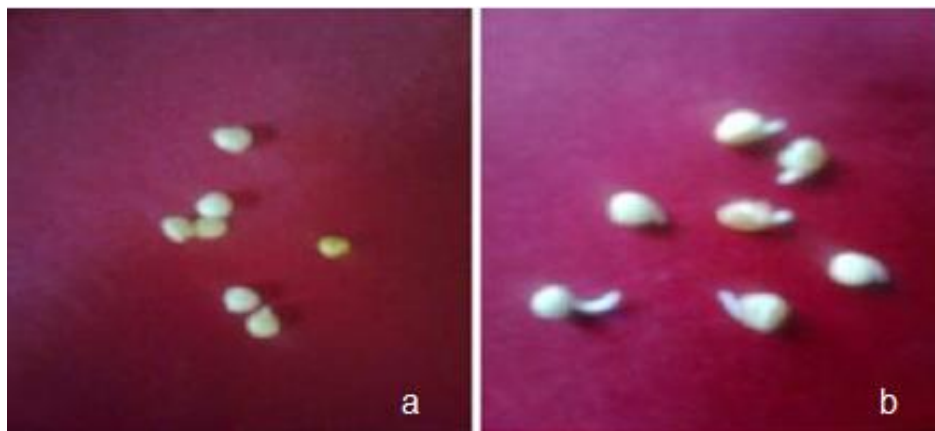
Figure 3. Effect of partially purified cytokinins on the weight of radish cotyledon seeds by *Pseudomonas* sp. control (a) and PN-4-SAN (b) after 3 days.

respectively. However, Lichter et al. (1995) analyzed different forms of cytokinins that is, zeatin, zeatin riboside, isopentenyladenin and two immunoreactive zeatin type compounds in the culture supernatant of pathogenic strain *Erwinia herbicola* and quantified zeatin in range of 200 to 300 ng/ml and zeatin riboside 13 to 107 ng/ml. So the selected *Pseudomonas* isolates were found to be quite efficient for the production of cytokinins like substances.

We have checked partially purified cytokinins like substance by thin layer chromatography having Rf value 0.72 to 0.75 (Table 2 and Figure 2) and radish cotyledon test (Table 3 and Figure 3). Hussain and Hasnain (2009) studied the production of cytokinins like substances using chromatography and bioassays methods and however, they found highest biological activity exhibited in substances located at the R_f 0.5, 0.54 and 0.58 in *Bacillus licheniformis*, *Bacillus subtilis* and *P. aeruginosa* as cytokinins production was found to be less in comparison of our findings. These results, therefore, indicate that *Pseudomonas* sp. can be used as a potent bacterial source to obtain high yields and provide a good alternative for cytokinins biosynthesis. As compared to

cytokinins obtained from other alternatives, they are less expensive and less time consuming. So, in future these be used for the commercial production of cytokinins.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Antibacterial susceptibility spectrum of some gram negative bacteria from suspected Otitis media patients

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Otitis media (OM) is a multifactorial disease characterized by high rate of recurrence in young children and considered to cause hearing impairment in children. This study was undertaken to evaluate the antibacterial sensitivity of Gram-negative bacteria of OM to some antibiotics. A total of Fifty four (54) samples were obtained from patients with suspected cases of otitis media coming into the National Ear Care Centre for the first time. Fifty four (54) patients (29 male, 25 female), with mean ages of 3.86 and 3.29 respectively, tested positive to OM pathogens with a total of 84 bacterial isolates while no culturable pathogen was observed in 4 patients (7.4%). The study reveals highest frequency of *Pseudomonas aeruginosa* 60 (71.43 %) followed by *Proteus mirabilis* 14 (16.67), *Klebsiella pnuemoniae* 6 (7.14) and *Escherichia coli* 4 (4.76). *P. aeruginosa* had the highest prevalence among patients < 30 years, whereas *K. pnuemoniae* and *E. coli* had the least isolated across all age groups. Antimicrobial susceptibility test showed highest frequency of resistance among all isolates to amoxicillin, cotrimoxazole, nitrofurantoin and nalidixic acid. However, gentamicin, ofloxacin, augmentin and tetracycline were effective against *Pseudomonas aeruginosa* but ineffective against other isolates. Although antibiotics are the most preferred and prescribed drugs in incidents of OM, it is clear from this study that antimicrobial resistance still remains a persistent among bacterial pathogens of otitis media.

Key words: Otitis media, antibiotic susceptibility, bacteria isolates.

INTRODUCTION

The incidence and chronicity of otitis media is increasing and likewise the effort in the design and development of both topical and systemic antibiotics due to the emerging resistance of bacteria to antimicrobial agents. Otitis media is associated with the inflammatory conditions of the mucosal lining of the middle ear resulting from middle ear microbial infection. The distinguishing features in the structure and anatomy of the ears, the length of the Eustachian tube made up of more flaccid cartilage and its anterior positioning, as well as compromised immunity;

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accounts for the higher prevalence of otitis media in children than in adults (Alsaimary et al., 2010; Weiner and Collison, 2003).

Factors that promote greater adhesion of bacteria to epithelial cells in children than adults includes; ignorance, poverty, poor environmental and personal hygiene. Similarly, socioeconomic burden, anthropogenic activities, as well as poor crowd control, non-compliance to treatment and inadequate health care diagnostics all represent possible predisposing and a perpetuating risk factors for the higher prevalence of otitis media in some developing and less developed countries (Kumar and Seth, 2011; Li et al., 2001; Pereira et al., 2004). Other burden of individual risk factors have been highlighted to relate to the interplay between the microbial load in the middle ear fluid and host factor immune response as well as the health-related behaviour of parents in the care of their young ones (Haggard, 2008; Rovers, 2008).

Researches have revealed OM to constitute an infection of the middle ear with manifestation of complex infective and inflammatory conditions. Based on differences in their presentation and treatment, OM are grouped as acute OM (AOM), OM with effusion (OME), chronic suppurative OM (CSOM) and cholesteatoma with associated complications as mastoiditis, meningitis, brain abscess formation, and sigmoid sinus thrombosis (Qureishi et al., 2014).

Dysfunction of the Eustachian tubes and bacterial infection are major relevant factors for chronic OM with gram-negative bacteria infecting the ear canal. However, fungi and viruses are persistent in some cases (Osazuwa et al., 2011). The normal flora of the skin also causes OM infection, with microbial pathogens gaining entry through acute perforation of the middle ear cleft via the Eustachian tube. Most frequently isolated pathogens associated with chronic and mixed infections in OM patients includes; *Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis, Staphylococcus aureus, Staphylococcus pseudintermedius, Streptococcus pyogenes, Pseudomonas aeruginosa, Proteus mirabilis, Klebsiella pneumoniae*, *Escherichia coli*, *Pasturella* spp., *Citrobacter* spp., *Enterobacter* spp and *Chlamydia pneumoniae* (Abera and Kibret, 2011; Block 1997). Among the causative pathogens, *E. coli, Klebsiella* spp. and *P. aeruginosa,* are the most resistant and prevalent. However, different antibiotics have shown to have an effective spectrum of activity towards both Gram-negative and Gram-positive pathogens (Sharma et al., 2004). Increasing frequency of bacterial resistance to antibiotics due to indiscriminate administration and use of antibiotics, poor surveillance of patients, inadequate clinical assessment and evaluation and resource-limited settings with inadequate infection control measures have resulted in creating concerns in the management strategy of OM infections.

The rural or grassroots communities in Nigeria have recorded large tales of OM infection.

The *in vitro* antibiotic activity against these causative pathogens and the antibiotic regimen are important in the choice of antibiotics for treatment of OM (Block, 1997).

Dearth of health personnel, inadequate primary health stations and poor diagnostic laboratories are factors militating against efficient care for OM patients in the rural and urban areas in Nigeria. Most cases in northern Nigeria involve prescription of antibiotics empirically to patients with OM in outpatient clinics or patent medicine stores without microbiologic evaluation (Musa et al., Bemu et al., 2015). The National Ear Care Centre Kaduna established to treat cases of OM patients in both rural and urban areas.

This study was undertaken to evaluate the antimicrobial sensitivity of some bacterial pathogens of otitis media among some patients without any record of treatment possible at the National Ear Care Centre, Kaduna, Nigeria.

MATERIALS AND METHODS

Specimen collection and sample size

The study population consisted of 54 patients (29 males and 25 females) between the ages of 0 and 69 years, visiting the clinic for the first time with cases of middle ear infection/or acute otitis media or any complaints of ear problems from June, 2014 to November, 2014. Randomized sampling design was employed. Total of 54 samples of aural discharge were collected with sterilized cotton swabs with the help of the attending physician. All clinical samples were transported in a Stuart transport medium to the microbiology laboratory of the Department of Microbiology Kaduna State University Nigeria within 1 h of collection. Ethical clearance was obtained from the National Ear care centre and with the informed consent of the patients and approval from the clinic management.

Isolation and identification of bacteria

Collected swabs were plated on MacConkey agar, blood agar and chocolate agar plates. MacConkey agar and blood agar were incubated aerobically, whereas chocolate plate was incubated anaerobically at 37°C for 24 h. Isolates were identified by colony morphology, gram staining reaction, catalase test, coagulase test, oxidase test, triple Sugar Iron agar (TSI), citrate utilization test, urease test, motility indole lysine (MIL) and methyl-Red Vogues Proskauer test (MR-VP) with glucose fermentation test (Cheesbrough 2006).

Antimicrobial susceptibility testing

Disk diffusion assay and agar plate dilution was performed according to NCCLS to assess theantibiotic resistance/susceptibility pattern of bacterial isolates. Bacterial suspension was prepared based on the McFarland standard solution 0.5 and inoculated onto Muller Hinton agar (Oxoid). For the disc diffusion technique, dics containing appropriate antibiotics (BBL™ UK), selected among the most commonly used, both with and without prescription - (amoxicillin (30µg), cotrimoxazole (30 µg), nitrofurantoin (100 µg), nalidixic acid (30 µg), gentamicin (10 µ)ofloxacin (1 µg), augmentin (30 µg) and tetracycline (30 µg)) - were tested against each isolate.

Table 1. Distribution of positive suspected cases of OM among patients visiting a clinic, June to November 2014.

Ages (years)	Male	Female	Total
0-9	18	10	28
10-19	5	8	13
20-29	2	3	5
30-39	1	0	1
40-49	0	1	1
50-59	1	0	1
60-69	0	1	1
Total	**27 (54%)**	**23 (46%)**	**50**

Results were reported as susceptible, intermediate or resistant, according to Clinical Laboratory Standards Institute's (CLSI 20) guidelines.

Data analysis

SPSS version 22 software was used for ANOVA Analysis of data and p value of <0.05 was considered statistically significant.

RESULTS

A total of 54 patients, representing 30 males and 24 females with age group of 0 to 69 years, and mean age (35.28, 3.85±6.4 SD and 3.28±4.0 male and female respectively) were investigated in this study. Based on the cultured swab, a total of 50 (92.59%) patients were suspected to have positive cases of OM while 4 (7.4 %) were negative (Table 1). 84 total isolates were obtained and *P. aeruginosa* was the most prevalent, representing 71.43%, *P. mirabilis* 14 (16.67%), *K. pneumoniae* 6 (7.14%) and *E. coli* 4 (4.76%) (Table 2). No significant difference between the genders in terms of cases recorded for OM among those attending the clinic, although there was a male predominance (27 (54%)) over females (23 (46 %)) in terms of positive cases based on the isolation pattern of bacterial isolates. Patients under the age of 20 years had higher identifiable cases recorded (Table 3). Lower incidence of positive cases was recorded for patients above 30 to 70 years.

Antimicrobial susceptibility

The susceptibility pattern of different bacterial isolates tested against eight antibiotics, gentamicin, augmentin, tetracycline, ofloxacin, nitrofurantoin, amoxicillin, nalidixic acid and cotrimozaxole (Table 4) revealed *P. mirabilis, E. coli and K. pneumoniae* have the highest resistance profile (64.24, 125 and 99%, respectively) as compared to other bacterial isolates. About 100% of all isolates

Table 2. Prevalence of bacterial agents of otitis media. among patients visiting a clinic, June to November 2014.

Organisms isolated	Number of isolates (%)
Pseudomonas aeruginosa	60 (71.43)
Proteus mirabilis	14 (16.67)
Klebsiella pneumoniae	6 (7.14)
Escherichia coli	4 (4.76)
Total	**84**

were resistant to amoxicillin and cotrimoxazole, while isolates showed sensitivity towards nitrofurantoin, nalidixic and gentamicin (64.3, 32.14 and 104.7, respectively). Only *P. aeruginosa* showed sensitivity to augmentin and tetracycline. *P. mirabilis* isolates showed higher frequency of resistance (100%) to amoxicillin, cotrimoxazole, augmentin and tetracycline. With regards to *E. coli* all isolates showed 100% resistance to amoxacillin, cotrimoxazole, augmentin and tetracycline.

DISCUSSION

The care of the ear is sacrosanct for the overall well-being of every individual. Hearing impairment is imminent in almost all age groups in some parts of Nigeria where lack of medical outreach, poor nutrition and awareness have hampered positive reactions from infected individuals.

In this study, confirmed cases of OM were observed among patients under the age of 30 years. Although children under the age of 10 are mostly vulnerable to OM, studies have revealed that the majority of affected age groups with OM are below 30 years.

Fifty four (54) cases investigated in the study against the 100 cases in the study by Kumar and Seth (2011) suggest a rational observation that adults are also as vulnerable to OM infections as children because all are confronted with similar predisposing factors. Higher number of bacterial isolates were from children under the age of 20 years in contrast to that recorded by Wasihun and Zemene (2015) where higher isolates came from children under age 0 to 5 years; although the study took into account the various stages of infection, not considered in this study.

Pseudomonas aeruginosa was the most isolated pathogen in almost all samples. As often found in all cases of chronic otitis media, *P. aeruginosa* is the most commonly isolated infective pathogen (Adoga et al., 2011; Nwabuisi and Ologe, 2002). The preponderance of *P. aeruginosa* among patients of all sexes and almost all age groups, suggests the diversity of its aetiology across geographical region as a predominant causative agent of otitis media as established in other reported findings across Nigeria and other parts of the world (Kumar et al.,

Table 3. Distribution of bacterial isolates among different age groups of patients visiting a clinic, June to November 2014.

Ages (years)	*Pseudomonas aeruginosa*	*Proteus mirabilis*	*Escherichia coli*	*Klebsiella pneumoniae*
0-9	30	9	4	3
10-19	11	3	2	1
20-29	8	2	0	0
30-39	6	0	0	0
40-49	3	0	0	0
50-59	2	0	0	0
60-69	0	0	0	0
Total	**60**	**14**	**6**	**4**

Table 4. Antibiotic sensitivity pattern of bacteria isolates among patients visiting clinic June to November 2014.

Antibiotics	*P. aeruginosa* (%) (%)	*K. pneumonia e* (%)	*E. coli* (%)	*P. mirabilis* (%)
Amoxicillin	0	0	0	0
Cotrimoxazole	1.6	0	0	0
Nitrofurantoin	0	50	0	14.3
Nalidixic acid	0	0	25	7.14
Gentamicin	25	33.3	25	21.4
Ofloxacin	26.6	16.6	75	21.4
Augmentin	3.3	0	0	0
Tetracycline	22	0	0	0

2013; Madana et al., 2011; Orji and Dike, 2015). Although not isolated in this study, infective pathogens such as *S. aureus* have also been isolated as predominant next to *P. aeruginosa* bacterial agent of OM (Prakash et al., 2013; Sharma et al., 2004).

The higher incidence of OM recorded in the study correlates with other reported incidence whereby male predominance is quite appalling than in female (Abubakar et al., 2014; Kumar et al., 2013). No rational explanation have been given for the variation in such prevalence, but it is asserted that over 80% of male experience at least one episode of AOM with concomitant hearing impairment at some stage, which impedes performance (Sambo et al., 2015).

Proteus mirabilis was higher in samples collected from patients between the age of 0 and 9 years and the most prevalent following *P. aeruginosa*, which is in tandem with previous findings by Kumar et al. (2013). Coliforms such as *K. pneumoniae* and *E. coli* were isolated from patients under age 20 with 11.9% occurrence. These pathogens have ubiquitous presence especially in faecal contaminated environment. Their widespread presence in almost all cases reported for OM suggests that they may have a greater pathogenic potential and it is a reflection of their relative high numbers due to poor environmental hygiene. Although the frequency of the isolated coliforms is lower than that reported in other findings, it has also

been reported to be the second most commonly isolated pathogenic agent among patients with chronic Otitis media (Afolabi *et al.,* 2012).

Similar study from the National Ear Care Centre revealed the predominance of *K. pneumoniae* and *E. . coli* as the most isolated among patients within the period under review (Bakari et al., 2011). This variation in its prevalence is attributable to the large number of patients investigated in contrast to that reported for this study. *Pseudomonas* sp and *Klebsiella* sp have also been isolated among patients with otitis externa in a recent study from the same clinic (Musa et al., 2015).

The leading role of the gram-negative bacteria such as *Pseudomonas* sp. *Klebsiella* sp. *E. coli*, and *P. mirabilis* in chronic otitis media, makes the choice of the correct antibiotics essential for treatment crucial (Adoga et al., 2011; Wasihun and Zemene, 2015). In most incidences, self-medication and unregulated dosage of antibiotics without laboratory counsel creates problems for treatments since prior attempt by patients to initiate antibiotics therapy can cause irreversible modifications to the pathogenic flora leading to changing patterns in their susceptibility to antibiotics (Park et al., 2008).

In-vitro drug sensitivity pattern of all isolates shows a very disturbing trend of resistance against tested antibiotics. Antimicrobial sensitivity test (AST) based on some selected antibiotics showed gentamicin, ofloxacin

and tetracycline to some extent effective against *P. aeruginosa*. ,Whereas Alsaimary et al. (2010) reported the effectiveness of amoxicillin to *P.aeruginosa*, which contrasts with the observation made in this study. Similarly, only gentamicin and ofloxacin were effective against *P. mirabilis*, *K. pneumoniae* and *E. coli*. Sensitivity pattern for other groups of antibiotics used in this study revealed higher resistance with *K. pneumoniae* and *E. coli* being the least sensitive in contrast to findings by Orji and Dike (2015).

Arbitrary selection of antibiotics in this study was based on the emerging concern on the diversity of microbial profile and AST pattern of most pathogens implicated in COM. Among the commonly available ones used as topical ear drops and investigated in this study, gentamicin was the only effective antibiotics against all isolated pathogens, which is reported to be effective against some commonly isolated organisms in the treatment of COM (Prakash et al., 2013). Arguably, it is proposed with good reasons why gentamicin could be toxic to patients with renal impairment (Ogbogu et al., 2013).

In most, cases, single or multiple antibiotic therapies are administered and the pattern of administration could be oral, parenteral and topical.

Amoxicillin, tetracycline and cotrimoxazole are the most commonly prescribed categories of antibacteria done singly or in combination. Other classes of prescribed antibiotics constitute the quinolones, penicillins and the nitroimidazoles with oral and topical quinolones administered to children (Abubakar *et al.,* 2014; Ain *et al.,* 2010).

In this study, these antibiotics were not effective against all isolates except for tetracycline with a descriptive pattern of sensitivity against 22% of *P. aeruginosa*. The high frequency of resistance displayed by *P. aeruginosa* and other isolates against tested antibiotics can indeed be ultimately linked to the notoriety of some pathogens in developing resistance when prescribed standards for administration of antibiotics are not adhered to (Okeke et al., 1999).

Similar study conducted recently, showed 78.3% of all gram-negative isolates were multidrug resistant and hence not sensitive to all antibiotics tested (Wasihun and Zemene, 2015). This report is also in tandem with this study findings where, although no MDR test was conducted, isolates showed resistance to almost all tested antibiotics. The variation in resistance for all isolates as reported in these findings underscores the task of selecting appropriate antibiotics for treatment since antibiotics seem to be most beneficial in acute otitis media (Rovers et al*.,* 2006).

Consequently, properties that confer resistance to most gram-negative bacterial pathogens such as membrane impermeability and the apparent persistence of pathogens in middle ear fluid causes higher rates of clinical OM that are mostly difficult to be eradicated by

initial antibiotic treatment (Leibovitz, 2008). The half-life of these antibiotics found in the environment where these pathogenic bacteria are transmitted via contamination, lingers for a long time after treatment thereby altering the microbial ecology in terms of resistance and bacterial susceptibility (Levy, 2002).

Conclusion

This study reports the antibacterial prevalence of OM infection cases among patients without previous records with the Ear Care Centre. The findings provide evidence that indeed OM persists among most children and adults below 30 years. Despite the high frequency of resistance based on the susceptibility profile of isolates, antibiotics such as gentamicin, ofloxacin and tetracycline remains first line of treatment as recorded in this study. These antibiotics remain beneficial in reducing the risks of hearing impairment and bacteriological eradication in the prevention of subsequent OM episodes. However, our study have also shown that resistance to some antimicrobial drugs by pathogens may develop. Reduction in the indiscriminate use of such antimicrobial agents by creating awareness among patients and preparation of variable antibiotic concentrations may limit the development of resistant bacteria.

Conflict of interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Nosocomial infection caused by multidrug resistant *Enterobacteriaceae* and their spread in inanimate surfaces in East-Algerian hospitals

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The inanimate hospital environment may serve as a reservoir for resistant bacteria that pose nosocomial infection risks often originating from cross contamination and the most common means of pathogens transference occurs between hands of health professionals, hospital equipment and patients. The aim of this study was to investigate various nosocomial infections with multidrug resistant *Enterobacteriaceae* (MDR-*E*) and their dissemination in hospital surfaces of two Algerian hospitals from January 2014 to December 2014. *Enterobacteriaceae* isolated from hospitalized patients and inanimate surfaces were identified by microbiological methods and confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALD-TOFMS). Antibiotic susceptibility was performed using disk diffusion method. Among 74 nosocomial infections detected, 44 were caused by MDR-*E* (59%) from different clinical specimen; 23 *Klebsiella pneumoniae*, 13 *Escherichia coli*, 5 *Enterobacter cloacae* and 3 *Citrobacter freundii*. From inanimate surfaces, MDR-*E* represents 61% (23 strains out of 38 MDR bacteria isolated); 9 *E. cloacae*, 8 *K. pneumoniae*, 4 *C. freundii* and 2 *E. coli*. In total, 67 MDR-*E* were isolated in 2014. Most *Enterobacteriaceae* show resistance to 13 antibiotics tested out of 15, especially to third-generation cephalosporins, thus resistance to all β-lactams except carbapenems. Here, the dominance of MDR-*E* isolated from nosocomial infections and in hospital surfaces in Algeria and a characterization of *Enterobacteriaceae* strains isolated from different specimens according to their species by MALDI-TOF MS were reported. Interestingly, high level of similarity was found between clinical and environmental strains in antibiotic resistance patterns.

Key words: *Enterobacteriaceae,* multidrug resistance, nosocomial infections, hospital surfaces.

INTRODUCTION

Nosocomial infections represent a significant health concern. About 60% of some of these infections involve multidrug-resistant (MDR) bacteria (defined as acquired non-susceptibility to at least one agent in three or more

antimicrobial categories) with an increasing predominance of Gram-negative organisms (Van Duijn et al., 2011; Magiorakos et al., 2012).

Hospitals are now more often facing the problem of antibiotic-resistant nosocomial infections, because of evolution and emergence of bacterial resistance to antibiotics and an increase in the number of immune-suppressed individuals worldwide, drug therapies and genetic disorders (Omenn, 2010).

Enterobacteriaceae are inhabitants of the intestinal flora and are among the most frequently isolated strains from hospitalized patients. They are the source of nosocomial infections. They have the propensity to spread easily between humans (hand carriage, contaminated food and water), causing infections such as cystitis and pyelonephritis with fever, septicemia, pneumonia, peritonitis, meningitis and device-associated infections (Nordmann et al., 2011; Paterson, 2006).

The emergence and spread of resistance in *Enterobacteriaceae* are complicating the treatment of serious nosocomial infections (Paterson, 2006). Current antimicrobial resistance profile of *Enterobacteriaceae* include the spread of non-susceptible strains to third-generation cephalosporins, recently, the emergence of carbapenem-resistant *Enterobacteriaceae* has been reported (Nordmann et al., 2011).

Enterobacteriaceae are supposed to be cross-transmitted like other nosocomial pathogens with the transient skin flora after direct contact with a colonized patient in the absence of hand hygiene. However, contamination of hands or clothes of healthcare workers could occur not only following direct contact with patients but also with the environment (Touati et al., 2008). Numerous nosocomial outbreaks of extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* have been reported worldwide (Touati et al., 2008). Also, their dissemination in hospital surfaces was reported (Jalapoor, 2011).

Previous study have detected extended spectrum of β-lactamases (ESBL) producing *Enterobacteriaceae* in environmental and clinical specimens in a surgery intensive care unit (Kac et al., 2004). In Algeria, several studies have shown the spread of multidrug-resistant *Enterobacteriaceae* (MDR-E) in hospitals (Baba Ahmad-Kazi et al., 2014). Recently, the presence of ESBLs enzymes in *Enterobacteriaceae* clinical and environmental isolates in Algerian hospitals was reported (Touati et al., 2008, 2010).

The present study was designed to investigate nosocomial infection with MDR-*E* and their spread in various inanimate areas of two East-Algerian hospitals in the same period.

MATERIALS AND METHODS

Sampling

The study was carried out from 01 January to 31 December 2014 in 2 hospitals in Guelma, East of Algeria. Nine wards were included in this study: emergency, operating room, pediatric, gynecology and neonatal, general surgery and general medicine in the hospital A; infectious diseases, phthisiology and hemodialysis in hospital B.

The present study was carried out on MDR-*E* strains isolated from patients hospitalized for at least 48 h, and from environmental samples (inanimate surfaces and adjacent equipment). Clinical strains included in this study were isolated from urine, pus, tracheal aspiration and blood, according to the diagnosis.

Environmental swabs samples were carried in inanimate area of approximately 10 cm² (patients beds, door's handles, patient's tables, treatment trolley, tap water, infusion stand, stretcher, siphon, radiator, treatment bench-top, drugs tray and nurse's hands) by means of a sterile swab moistened with nutrient broth medium (Algeria Pasteur institute) (Sehulster et al., 2003). After sampling, swabs were incubated in the nutrient broth medium at 37°C for 24 h, then; the strains were subcultured and isolated on Mac Conkey agar (Bio Rad).

Bacterial strains

The identification of bacteria was performed with microbiological methods (Gram stains, oxydase test and API20E identification system « bio-Mérieux ») (www.biomerieux.fr) and confirmed by matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF MS) method (Microflex; Bruker Daltonics) using 96 spot polished-steel targets (Seng et al., 2009). The peak profiles of identified strains were compared and analyzed using Biotyper 3.0 software (Bruker Daltonics) to build a dendrogram of mass spectral data (Bakour et al., 2012).

Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed according to the antibiotic susceptibility standard disc diffusion method on Mueller-Hinton agar as recommended by EUCAST (2013) (www.sfm-microbiologie.org/). The susceptibility of the isolates was determined using discs of amoxicillin (25 µg), amoxicillin-clavulanate (20/10 µg), ticarcillin–clavulanate (75/10 µg), céfoxitine (30 µg), aztreonam (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg), imipenem (10 µg), amikacine (30 µg), gentamicin (10 µg), ciprofloxacin (5 µg), trimithoprime-sulfamethoxazol (1.25/23.75 µg), rifampicin (30 µg), fosfomycine (50 µg) and colistin (50 µg) (Bio-Rad). The results were interpreted according to the recommendations of EUCAST (2013).

RESULTS

Bacterial isolates

Over the period of this study (January 2014 to December 2014), 112 multidrug-resistant bacteria were isolated, of which, 74 were isolated from different clinical specimen of nosocomial infections, and 38 were surfaces isolates. Sixty-seven of them (67/112) were MDR-*E*, where, 44 out of 74 (59%) were isolated from clinical samples: 23 *Klebsiella pneumoniae*, 13 *Escherichia coli*, 5 *Enterobacter cloacae* and 3 *Citrobacter freundii*. However, as for inanimate surfaces, 23 (61%) out of 38 MDR-*E* were identified: 9 *E. cloacae*, 8 *K. pneumoniae*, 4 *C. freundii* and 2 *E. coli*.

Majority of the strains were recovered from infectious

Table 1. The distribution of multidrug resistant *Enterobacteriaceae* isolated from clinical and surfaces samples according to the hospitals wards.

Wards	Clinical isolates n (%)	Surface isolates n (%)
Emergency[A]	0 (0)	3 (13)
Operating room[A]	0 (0)	0 (0)
Pediatric[A]	10 (23)	3 (13)
Gynecology and neonatal[A]	8 (18)	0 (0)
General surgery[A]	6 (14)	5 (22)
General medicine[A]	1 (2)	0 (0)
Infectious diseases[B]	13 (30)	6 (26)
Phthisiology[B]	5 (11)	5 (22)
Hemodialysis[B]	1 (2)	1 (4)
Total	44 (100%)	23 (100%)

n. Number; A. hospital A; B. hospital B.

Table 2. The distribution of *Enterobacteriaceae* isolates according to the clinical samples type.

Sample type	Isolates n (%)
Urine	38 (86)
Pus	3 (7)
Tracheal aspiration	2 (5)
Blood	1 (2)
Total	44 (100%)

Table 3. The distribution of multidrug resistant *Enterobacteriaceae* isolated from inanimate surfaces.

Surface	Isolates n (%)
Door's handles	5 (22)
Siphons	4 (18)
Taps water	2 (9)
Stretchers	2 (9)
Patient's beds	2 (9)
Patient's tables	2 (9)
Nurse's hands	1 (4)
Treatment trolley	1 (4)
Radiator	1 (4)
Treatment bench-top	1 (4)
Infusion stand	1 (4)
Drugs tray	1 (4)
Total	23 (100%)

diseases ward (19 MDR-*E* out of 67), of them, 13 (30%) were from patient samples, and 6 (26%) from inanimate surfaces (Table 1). On the other hand, urinary nosocomial infection was the predominant, urine was the source of 86% of MDR-*E* (38 strains) (Table 2). However, source of environmental samples have a close rate, of which, door's handles was the predominant with 22% (Table 3).

An MSP dendrogram was constructed using MALDI-TOF spectra of MDR-E isolates through Biotyper 3.0 software (Bruker Daltonics, Bremen, Germany). Four groups were found to correspond to a 4 cluster obtained at the arbitrary distance value of 100. Each cluster corresponds to a species, by grouping the clinical strains and those of the surfaces belonging to the same species (Figure 1).

Antimicrobial susceptibility test

The results of *in vitro* susceptibilities to 15 antimicrobial agents test for clinical and surfaces isolates of 4 common species of *Enterobacteriaceae*, revealed high-level resistance to antibiotics, especially to β-lactams, except imipenem, with 100% of resistance to amoxicillin/clavulanic acid, ticarcillin/clavulanic acid,

cefotaxime and ceftriaxone. The percentage of cefoxitine range from 41% for clinical strains to 65% for surfaces, and aztreonam, range from 82% for clinical to 92% for surfaces isolates.

From high to moderate resistance to trimethoprim-sulfamethoxazole, aminoglycosides, fluoroquinolones and fosfomycin were detected: 64 and 78% for trimethoprim-sulfamethoxazole from clinical and surfaces strains, respectively, and the rate of resistance to gentamicin ranging from 64 to 74% and 23 to 0% for amikacine, 48 to 44% for ciprofloxacin, and that of fosfomycine range from 52 to 44% for clinical and surfaces strains, respectively. A high level resistance to rifampicin was detected, 91% for both, clinical and surfaces strains. No resistance was detected for colistin (Figure 2). Interestingly, 76% of clinical and surfaces strains presented 100% of similarity in the antibiotic resistance patterns.
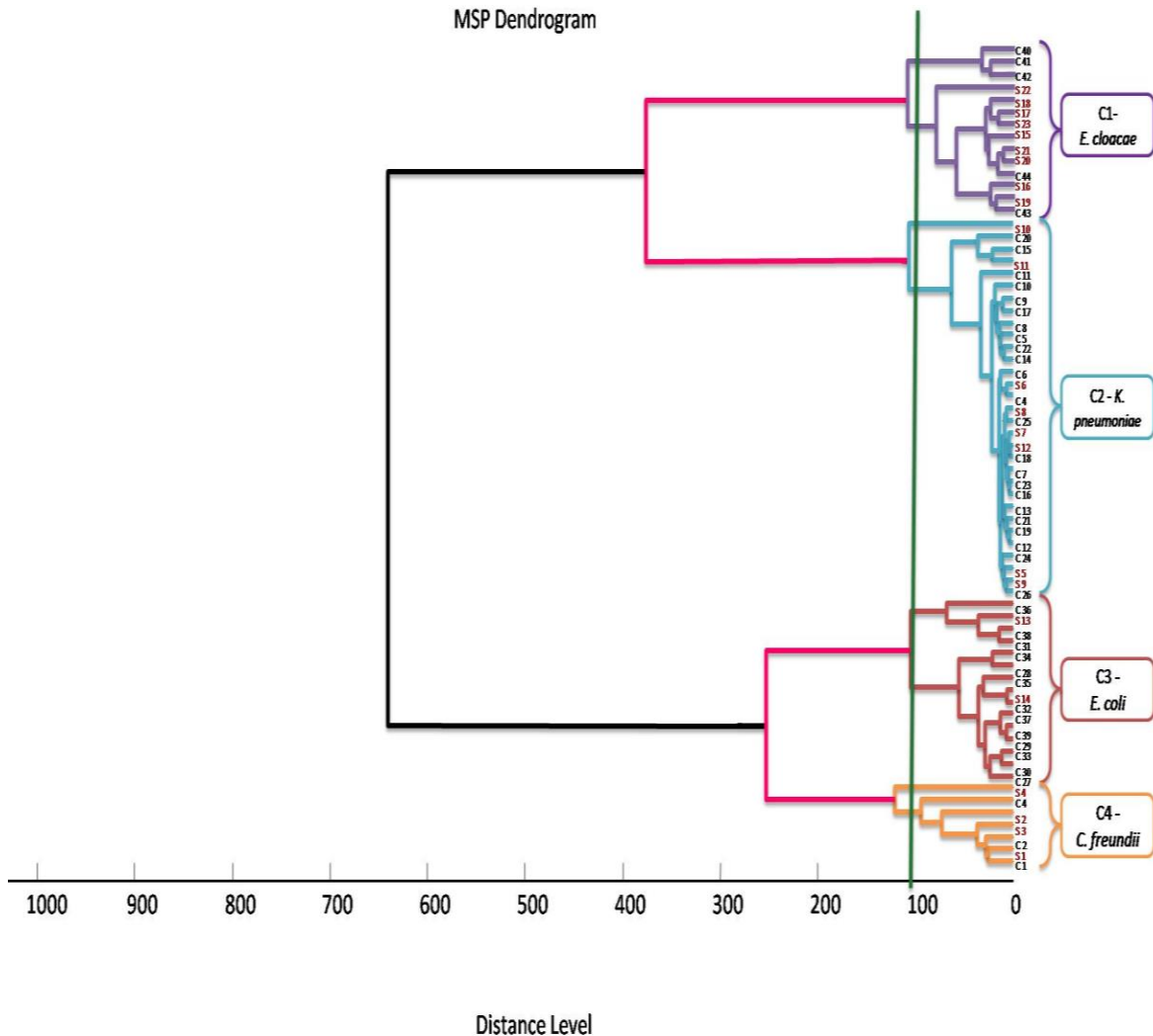
MSP Dendrogram

Figure 1. Cross-wise minimum spanning dendrogram generated by the Biotyper 2.0 program for the 67 MDR-*Enterobacteriaceae* clinical and surfaces strains isolated from East-Algerian hospitals (A and B). The arbitrary distance level at 100 is indicated as vertical green lines. Clustering of the strains according to this cut-off value significantly associates all *E. cloacae* strains in one cluster C1, *K. pneumoniae* in C2, *E. coli* in C3 and associates all *C. freundii* strains in C4. (Strains code: Black: clinical strains, Red surfaces strains).

DISCUSSION

Bacterial contamination of touch surfaces pose a potential risk of nosocomial infection, in Algeria. ESBL-producing *Enterobacteriaceae* in hospital surfaces and the clonal relatedness between clinical and surfaces isolates where previously reported (Touati et al., 2008, 2010). This study shows that multidrug resistant *Enterobacteriaceae* continue to be associated with hospital-acquired infections in Algerian hospitals.

Several studies reported the prevalence of *Enterobacteriaceae* as causative of nosocomial infections, in India; a recent study revealed that *Enterobacteriaceae* are the most frequently isolated in hospitals (Sahu et al., 2016), this concur with the high

frequency of *Enterobacteriaceae* (59%) revealed in the present study. In the present study, the proportion of surfaces contamination with MDR-*E* was 61%. This proportion is higher than the rates revealed in previous studies (9, 10.7 and 26%) (Touati et al., 2010; Kac et al., 2004; Jalapoor, 2011).

To the authors' knowledge, several studies demonstrate the rate of nosocomial infections and the contamination of hospital environment in intensive care units, pediatric and neonatal wards. In this study, the ward most affected by nosocomial infections with contamination of their surfaces was infectious disease ward.

This study has revealed that urinary nosocomial infection was the most detected; several studies have

Enterobacteriaceae
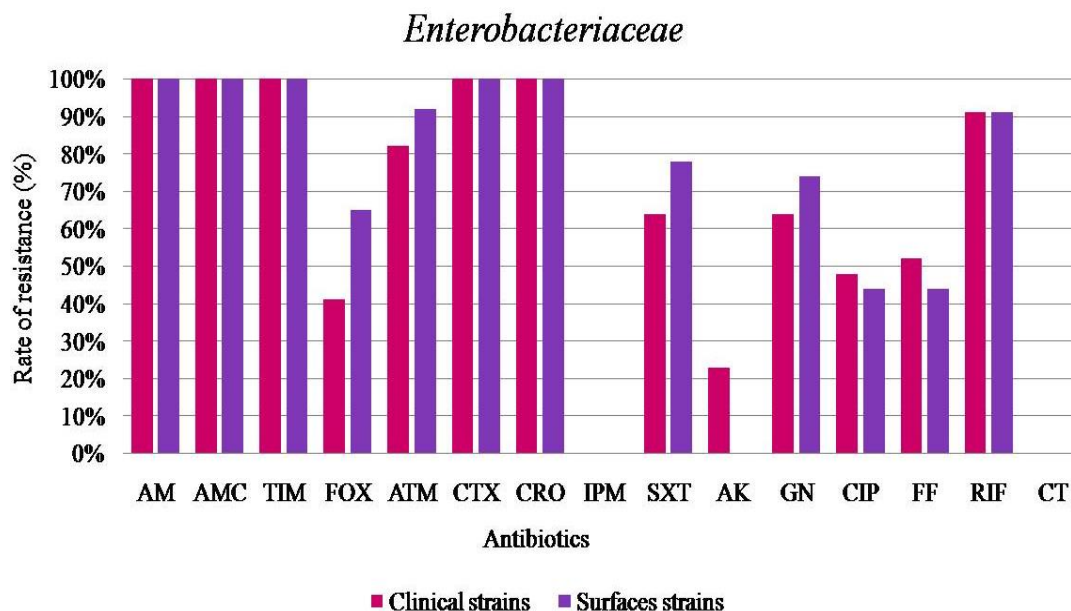
Figure 2. Antimicrobial resistance pattern among clinical and surfaces strains. AM. Amoxicillin, AMC. amoxicillin-clavulanate, TIM. ticarcillin–clavulanate, FOX. cefoxitine, ATM. aztreonam, CTX. cefotaxime, CRO. ceftriaxone, IMP. imipenem, SXT. trimithoprime-sulfamethoxazol, AK. amikacin, GN. gentamicin, CIP. ciprofloxacin, FF. fosfomycine, RIF. rifampicin, and CT. colistin.

shown 4 most frequent types of hospital-acquired infection: pneumonia, surgical site infection, urinary tract infection and bloodstream infection (Gaynes et al., 2005). Literatures have previously reported that door handles were the main source of bacteria and this is consistent with this study (De Abreu et al., 2014; Taneja et al., 2004). MALDI-TOF MS is mostly used as the main tool for species identification (Seng et al., 2010; Berrazeg et al., 2013). It is a rapid and alternative method for a better epidemiology survey of these bacteria, especially for a suspected outbreak and/or emergence of specific clones, in order to implement rapid infection control measures (Bakour et al., 2012)

Currently, some studies are focused on whether is possible to use MALDI-TOF MS as a discriminatory tool for typing (Mesli et al., 2013; Batah et al., 2015; Novais et al., 2014; Sachse et al., 2014).

The analysis of the dendrogram generated by Biotyper software showed that MALDI-TOF MS was useful to characterize the *Enterobacteriaceae* strains according to their species; the protein signatures formed 4 separate clusters related to each one the species. This is consistent with the recent findings where authors showed that MALDI-TOF MS is able to identify and class *Acinetobacter* species strains in separate clusters (Mesli et al., 2013). These results propose that the isolated strains of the same species either of clinical or environmental origin, are very close in their protein structure, it suppose that the grouping of species isolated from different wards of two hospitals could be due to either patient-to-patient, visitor to patients transmission or

to the contamination of nurse's hands and hospital surfaces and equipments.

In these series of strains, all *Enterobacteriaceae* presented high level resistance to most antibiotics tested, this results have been likely previously reported in several studies worldwide, either clinical or surfaces isolates (Karlowsky et al., 2003; Touati et al., 2010; Sahu et al., 2016; Jalapoor, 2011).

Another important finding in this study is that most environmental *Enterobacteriaceae* isolates have similar MDR profiles of strains isolated from hospitalized patients over the same period. All recent studies from different countries have shown increasing incidence of MDR clinical and surfaces isolates of *Enterobacteriaceae* (Kac et al., 2004; Touati et al., 2008).

Finally, contamination of hospital surfaces with multidrug resistant *Enterobacteriaceae* is a potential source for nosocomial infections which can be transmitted from patient to patient, hospital surfaces and equipments to patients, also from hospital staff to patients. Importantly, several articles demonstrate that enhanced cleaning, the use of no-touch methods for terminal room disinfection, and potentially the use of self-disinfecting surfaces may help in reducing nosocomial infections and contamination of hospital environment.

Conflict of interest

All the authors have declared that there is no conflict of interest.

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Full Length Research Paper

Cloning and characterization of an endo-β-1,4-xylanase gene from *Colletotrichum lindemuthianum* and phylogenetic analysis of similar genes from phytopathogenic fungus

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Colletotrichum lindemuthianum **is the etiological agent of anthracnose, one of the main diseases of bean (*Phaseolus vulgaris*). In this study, the complete cDNAs of two endo-β-1,4-xylanase genes (*xyl1*) from non-pathogenic (0) and pathogenic (1472) races of *C. lindemuthianum* were isolated and characterized. To get an insight into the role of endo-β-1,4-xylanases in their different lifestyles, *xyl1* gene expression and enzyme activity in mycelia of both races grown in the presence of xylan or *P. vulgaris* cell walls were investigated. The *xyl1* sequence analysis and Clustal alignment revealed the characteristic elements of genes coding for endo-β-1,4-xylanases of the GH11 family. The growth of the two races with glucose as the sole carbon source showed both basal transcription levels of *xyl1* and endoxylanase activity. When glucose was substituted with xylan or plant cell walls, *xyl1* transcription, and enzyme activity significantly increased in race 1472 as compared to race 0. The pathogenic race degraded xylan faster and grew better than the non-pathogenic counterpart. Seemingly, the regulation of xylanolytic gene expression, enzyme production and the nature of the assimilatory carbon substrates processed by these organisms play a determinant role in their lifestyle. Phylogenetic analyses of XYL1 and endo-β-1,4-xylanases from other fungi revealed a diversification process and separation of proteins from the same fungal species into different lineages.**

Key words: *Colletotrichum lindemuthianum*, *Phaseolus vulgaris*, endo-β-1,4-xylanase, gene expression, phylogeny.

INTRODUCTION

Colletotrichum lindemuthianum is an economically important phytopathogen and together with its host *Phaseolus vulgaris*, represents a convenient model for studying the physiological and molecular basis of plant-pathogen interactions (Dean et al., 2012; Perfect et al.,

1999). This species encompasses different strains or special forms known as races, physiological races or pathotypes identified through the interaction with a group of 12 different cultivars of *P. vulgaris*, a system used worldwide (Rodríguez-Guerra et al., 2006). A non-

pathogenic race and more than 100 pathotypes with different virulence levels have been reported around the world. AFLP analyses of 10 out of the 54 *C. lindemuthianum* pathotypes identified in México have shown high genetic diversity with several lineages (Gonzalez et al., 1998; Sánchez-García et al., 2009). *C. lindemuthianum* is an intracellular hemibiotrophic whose physiological races invade the plant in a manner consistent with the gene-for-gene model interactions (Flor, 1971; Oblessuc et al., 2012). Monogenic dominant resistance in common bean cultivars leads to the appearance of localized necrotic spots that are typical of the hypersensitive response (HR) (O'Connell and Bailey, 1988). After penetrating a host epidermal cell in a susceptible cultivar, pathogenic races of the fungus develop an infection vesicle and extend into adjacent cells by means of large primary hyphae, which invaginate without penetrating the cell membrane and thus persisting as a biotrophic interaction (Münch et al., 2008; O'Connell and Bailey, 1988). Once a large area of the plant tissue has been colonized, necrotrophic hyphae develop. This step closely correlates with the production of a number of host cell wall degrading enzymes that are characteristic of phytopathogenic fungi (Dodds et al., 2009; King et al., 2011; Wijesundera et al., 1989). Currently, race 0 is one strain of *C. lindemuthianum* unable to infect 12 different cultivars of *P. vulgaris*, which contrasts with race 1472, one virulent isolated in México (Rodríguez-Guerra et al., 2006). This difference makes the comparison of the two races a convenient approach to investigate the role played by host cell wall degrading enzymes in the pathogenicity of *C. lindemuthianum*.

Analysis of genomic sequences from plant saprophytic and pathogenic fungi has led to the identification of putative genes encoding for carbohydrate-active enzymes (CAZymes) involved in the degradation of plant cell wall. Comparison of these genes has contributed to our understanding of their lifestyle and helped to create infection models (Zhao et al., 2013). For example, biotrophic fungi tend to have fewer CAZymes than necrotrophic and hemibiotrophic fungi. Saprophytic fungi have fewer CAZymes than plant pathogenic fungi, and dicot pathogens often contain more pectinases than monocot pathogens (Zhao et al., 2013). Nevertheless, there have been few studies examining the genetic expression and enzymatic activity of these CAZymes as compared to the vast diversity of substrates presented by hosts.

Endoxylanases are CAZymes produced by some saprophytic and pathogenic fungi (Polizeli et al., 2005; Sunna and Antranikian, 1997) and are responsible for the depolymerization of xylan in plant cell wall (Collins et al., 2005; van den Brink and de Vries, 2011). There are currently more than 100 families of glycoside hydrolases (GHs) in the CAZymes database (Cantarel et al., 2009; Lombard et al., 2014) (http://www.cazy.org/). Endoxylanases are distributed in families GH10 and GH11, which correspond to the F and G families, respectively (Ahmed et al., 2009; Biely et al., 1997; Gilkes et al., 1991). The endo-β-1,4-xylanases (EC 3.2.1.8) belonging to family GH11 hydrolyze the β-1, 4 bond of xylan generating xylooligosaccharides, which are further hydrolyzed by β-xylosidase to xylose units (EC 3.2.1.37) (Biely, 1985; Pollet et al., 2010). These endoxylanases fold into β jelly roll sheets that define their secondary structure (Paës et al., 2012). Phylogenetic analysis of endoxylanases of plant saprophytes and pathogens can contribute to the understanding of the evolutionary process in relation to host types and different invasion/nutritional strategies (biotrophic, necrotrophic, or hemibiotrophic).

On this background, here, for the first time, the isolation and characterization of *xyl1* cDNA, which encodes an extracellular endo-β-1,4-xylanase in non-pathogenic (0) and pathogenic (1472) races of *C. lindemuthianum* was reported. Moreover, to understand the role of β-1,4-xylanase in the different fungal lifestyles, *xyl1* gene expression and endoxylanase activity in mycelia of both races grown in the presence of xylan or *P. vulgaris* cell walls were investigated.

Finally, the results of Clustal alignment and phylogenetic analyses of XYL1 from *C. lindemuthianum* and similar enzymes reported in other species of fungi are also presented.

MATERIALS AND METHODS

Strains and culture conditions

C. lindemuthianum races 1472 and 0 were kindly provided by Dr. June Simpson (CINVESTAV-IPN, Unidad Irapuato, México), which were reported and characterized by interaction with differential varieties of bean (*Phaseolus vulgaris*) and molecular strategies (RAPD and AFLP) as the pathotypes 1472 and 0 by González et al. (1998) and subsequently analyzed by Rodríguez-Guerra et al. (2006). *C. lindemuthianum* was maintained on potato dextrose agar (PDA) (Difco, México) at 20°C. For expression analysis, 1.6 mg (approximately 5 cm^2) of mycelia from both races was inoculated into 250 mL-Erlenmeyer flasks containing 50 mL of PD medium and shaken (150 rpm) at 20°C. After 9 days, mycelia was collected by filtration, washed with water and transferred to 125 mL-Erlenmeyer flasks containing 50 mL of modified Mathur's medium (Acosta-Rodriguez et al., 2005), supplemented with 2.5% of glucose, xylan (from beechwood; Sigma-Aldrich, St. Louis, MO, USA) or cell walls from *P. vulgaris* (cv. Flor de Mayo). Flasks were shaken (150 rpm) at 20°C and after various periods of time, mycelia were collected by filtration, washed with water and stored at -80°C until use.

For enzyme analysis, 125 mL-Erlenmeyer flasks containing 50 mL of modified Mathur's medium (Acosta-Rodriguez et al., 2005)

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Table 1. Sequences of endoxylanases used for the analyses.

Fungi	GenBank access	In this study	References
Didymella pisi	CAA93120.1	*Didymella_pisi*	(Lübeck et al., 1997a)
Botrytis cinérea	AAZ03776	*B.cinerea_xyn11A*	(Brito et al., 2006)
Cochliobolus carbonum	L13596	*Cochliobolus_carbonum-xyl1*	(Apel-Birkhold and Walton, 1996)
Cochliobolus carbonum	U58916	*C.carbonum-xyl3*	(Apel-Birkhold and Walton, 1996)
Cochliobolus sativus	CAA06151.1	*C. sativus-xyl2*	(Emami and Hack, 2001)
Claviceps purpurea	CAA76570	*Claviceps_purpurea-xyl1*	(Giesbert et al., 1998)
Fusarium oxysporum f. sp. lycopersici.	AAK27975.1	*Fusarium_oxysporum-xyl4*	(Gomez-Gomez et al., 2002)
Fusarium oxysporum f. sp. lycopersici.	AF246830_1	*F.oxysporum-xyl5*	(Gomez-Gomez et al., 2002)
Helminthosporium turcicum	CAB52417.1	*Helminthosporium_turcicum-htxyl1*	(Degefu et al., 2004)
Helminthosporium turcicum	CAD70174.1	*H. turcicum-htxyl2*	(Degefu et al., 2004)
Magnaporthe grisea	L37530	*Magnaporthe_grisea-Xyn22*	(Wu et al., 1995)
Lentinula edodes	AAL04152.1	*Lentinula_edodes*	(Lee et al., 2005)

supplemented with one of the carbon sources described above, were inoculated with 1.6 mg dry weight (approximately 5 cm^2) of a 9-day-old colony grown on PDA and incubated at 20°C with continuous shaking (150 rpm). After different periods of time, cultures were centrifuged at low speed and the mycelia and supernatants were saved. Fungal growth was measured as mg of wet mycelia except in those experiments where plant cell walls were used as carbon sources. In these cases, growth was measured as the amount of mycelial protein, as residual undegraded cell walls interfered with weight quantification. A 3-mL aliquot of the cell-free supernatant was filtered through a column (1.5 x 6 cm) of Bio-Gel P-6 (Bio-Rad, Hercules, CA, USA), equilibrated and eluted with 50 mM sodium acetate buffer, pH 5.0 (buffer A), at 4°C to prepare the enzymatic fraction. Fractions corresponding to the void volume (V_o) were pooled and the pool, labeled as the filtered extracellular medium (FEM), was used to determine protein and enzyme activity.

Preparation of plant cell walls

P. vulgaris seedlings (cv. Flor de Mayo) were grown for seven days, and cell walls were extracted and purified from hypocotyls by washing in organic solvents as described elsewhere (Fry, 2006).

RNA isolation

Total RNA was purified from mycelia using the Sokolovsky method (Sokolovsky et al., 1990). RNA samples were treated with DNAse I according to the manufacturer's instructions (Invitrogen, Grand Island, NY, USA) to eliminate DNA. The quality and concentration of total RNA were assessed using a Biophotometer Plus system (Eppendorf, Barkhausenweg, Hamburg, Germany).

cDNA isolation, sequencing and analysis

A cDNA fragment (223 bp) of the endo-β-1,4-xylanase gene (*xyl1*) from race 1472 of *C. lindemuthianum* was amplified using the reported primers XYNG2-F [5´-GA(A/G)TA(T/C)TA(T/C)AT(T/C/A)GT(A/T/G/C)GA(A/G)(A/T)(G/C)(A/C/G/T)TA-3´] and XYNG2-R [5´-GCCCA(A/C/G/T)GC(A/G)TT(A/G)AA(A/G)TG(A/G)   TT-3´] according to Kimura (Kimura et al., 2000). Total RNA was isolated from mycelia induced with xylan for 24 h. This fragment was

sequenced (data not shown), the specific primers Xyl-D110 (5'-GCGTGAACCAGCCCAGCATC-3') and Xyl-GSP1 (5'-CAGCGTTGAAGTGGTTG-3´) were designed. The cDNA of *xyl1* was amplified by 3´and 5´RACE as specified by the manufacturer using the SuperScript III First-Strand Synthesis System (Invitrogen, Grand Island, NY, USA) using total RNA isolated from mycelia of race 1472 induced with xylan for 24 h. Finally, the complete cDNAs of *xyl1* were amplified with the specific primers designed on the 5´ UTR, Clxil1-F (5´-ACTTATCATCGTCCGCTTCAACCA-3´) and 3´ UTR Clxil1-R (5´-GCAATCCTCGGAGTTCCAATCTGA-3´), using total RNA of mycelia from both races induced with xylan for 24 h. The PCR incubation mixture was heated at 96°C for 3 min in a Thermocycler (Eppendorf Master Cycler Gradient, Brinkmann, Westbury, NY), followed by 30 cycles of denaturation for 30 s at 96°C, annealing for 35 s at 60°C, extension for 1 min at 72°C and then by a final extension for 10 min at 72°C. All PCR products obtained from both races were ligated into the pCR 2.1 vector (Invitrogen).

The sequences of both strands of cDNA were determined by automatized sequencing using the dideoxy-chain termination method by the commercial service of Macrogen USA. Nucleotide sequences were analyzed using DNAsis (Hitachi), Mega6 (Tamura et al., 2013) and 4Peaks v 1.7.2 software (Griekspoor, 2012). The sequence of the N-terminal secretion signal was identified with SignalP 4.1 Server (Bendtsen et al., 2004). The protein molecular masses, pI values and *N*-glycosylation sites were calculated using ExPASy Proteomics Server (Wilkins et al., 1999). The nucleotide sequences of 12 endo-β-1,4-xylanases from fungal species were obtained from the NCBI GenBank (Table 1) and were numbered when more than one gene was present in a genome. Multiple sequence alignments were performed with Clustal X software (Larkin et al., 2007) using the default parameters. The signal peptide sequences and N-and C-terminal extensions were excluded.

Expression analysis of *xyl1*

Relative quantification of gene expression (RT-qPCR) was performed using the comparative Ct method (DDCt) on a Step One Plus Real-Time PCR System (Applied Biosystems Carlsbad, CA, USA) according to the manufacturer's instructions. Reactions were carried out with an SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA). A fragment of cDNA (208 bp) was amplified with the designed specific primers TRxil2-F (5´TGTCGGAGGCAAGGGCTGGAATC 3´) and TRxil2-R (5´

ATAAGTGCCGCCGTCGCTGGTGA 3´) using total RNA obtained from mycelia induced with 2.5% glucose for 8 h, or xylan or CW from *P. vulgaris* for 0, 2, 4, 6, 12, 24 and 48 h and 3, 4, 5, 7 and 9 days. Fragments of cDNA (372 and 339 bp for races 1472 and 0, respectively) from the *C. lindemuthianum* β-tubulin gene (*btub*) were amplified with the primers B36F and B12R (5´ CACCCACTCCCTCGGTGGTG 3´ and 5´CATGAAGAAGTGAAGACGCGGGAA 3´, respectively) (Thon and Royse, 1999) using total RNA obtained from mycelia grown with glucose. The β-tubulin fragments were sequenced and deposited in GenBank (Accessions: KF487130 and KM587706). The specific primers TR-βtub2-D and TR-βtub2-R (5´ GAATTCCCCGACC GTATGATG 3´ and 5´ CGGAGAGGGTGGCGTTGTA 3´, respectively) were designed and used as an internal control (endogenous gene).

Data were obtained from three independent experiments performed in triplicate, and analysis of variance (ANOVA) was carried out. The results are reported as the mean and standard errors (SE). *P* values <0.05 were considered significant.

Assay of endoxylanase activity

Endoxylanase catalyzes the hydrolysis of the 1,4-β-D-xylosidic linkages present in xylan, releasing β-D-xylopyranosyl oligomers and smaller molecules such as β-D-xylopyranosyl mono-, di- and tri-saccharides (Collins et al., 2005; Polizeli et al., 2005). These products can be quantified as reducing sugars using colorimetric methods. Accordingly, reaction mixtures containing 0.5 mL of 0.5% xylan (Sigma), 0.5 mL FEM and buffer A in a final volume of 1 mL were incubated at 30°C. After 10 min, the reaction was terminated by heating the samples in boiling water for 5 min and, after cooling, the amount of reducing sugars was measured using the Nelson-Somogyi method (Nelson, 1944). Activity was expressed, as µg of reducing sugars released in one min. Specific activity was referred to one milligram of protein.

Statistical analysis

Statistical analysis of data was performed using ANOVA with a GLM using a 2 x 3 factorial design (two races and three carbon sources) at each time point. The mean values of the enzyme activity of the fungus and its substrate and the amount of protein and mycelial protein were compared and grouped using the Tukey's test. All analyses were performed with STATISTICA v 10 software (Inc., 2013).

Phylogenetic analyses

Phylogenetic analyses were performed on the deduced amino acid sequence for *C. lindemuthianum* XYL1 and 11 endo-β-1,4-xylanases characterized from other ascomycetes (Table 1) and one sequence from the basidiomycete *Lentinula edodes* as an outgroup. Deduced amino acid sequences were aligned with Clustal X software (Larkin et al., 2007) using the default parameters. Before phylogenetic analyses, the signal peptide sequences, and N- and C-terminal extensions were excluded. Phylogenetic analysis was performed under Neighbor-Joining and Maximum likelihood criteria using Mega6 (Tamura et al., 2013). The JTT substitution model and gamma correction were used, 1000 bootstrap replicates were performed. The amino WAG evolution model with gamma correction was utilized for maximum likelihood analysis, and the most parsimonious trees were estimated using the heuristic search option (Nearest-Neighbor-Interchange-NNI) with random sequence addition (five random replicates). A WAG+G substitution model was used, and 1000 bootstrap replicates were performed.

RESULTS

Isolation and sequence analysis of *xyl1*

cDNA encoding an endo-β-1,4-xylanase was isolated from each race and deposited in GenBank (Accessions: KF487129, KM587707). The *C. lindemuthianum xyl1* cDNA of race 1472 has 905 bp, with a 5´ UTR of 25 bp and a 3´ UTR of 211 bp (Figure 1). The *xyl1* cDNA of race 0 has 751 bp, with a 5´ UTR of 23 bp and a 3´ UTR of 59 bp (Figure 2). At nucleotide and amino acid levels, the sequence of both races showed 100% identity. Comparison at amino acid level with corresponding sequences in GenBank showed 67, 64, 62 and 61% identity with a *xylI* of *P. tritici-repentis, xil1 of Cochliobolus carbonum, htxyl1* of *Helminthosporium turcicum*, *Xyn22 of Magnaporthe grisea,* respectively. The putative protein has an open reading frame of 222 amino acids with a signal peptide cleavage site between Ala^{19} and Ser^{20} (Figures 1 and 2), according to the SignalP 4.1 web server (Bendtsen et al., 2004), which is consistent with previously reported sequences (Apel-Birkhold and Walton, 1996; Kimura et al., 2000). The putative mature protein (residues 20 to 222) has a calculated molecular mass of 21.71 kDa and a pI of 8.94. A potential *N*-glycosylation site at Asn^{71} was found with the ExPASy Proteomics Server (Ellouze et al., 2011; Wilkins et al., 1999).

The multiple sequence alignment of the deduced amino acid sequences of *C. lindemuthianum* XYL1 with the endo-β-1,4-xylanases of other fungi revealed the conserved motif EYY where a residue corresponding to the catalytic site is found (Figure 3). In various bacteria and fungi, this motif was reported to be the key segment for enzyme catalytic activity or substrate binding (Apel et al., 1993; Degefu et al., 2001; Li and Ljungdahl, 1994). The deduced amino acid sequence of *C. lindemuthianum* XYL1 revealed two Glu residues (E^{96}, E^{187}) that are highly conserved in xylanases of family GH11 and are likely to be involved in hydrolysis of the glycosidic bond (Figures 1, 2 and 3) (Degefu et al., 2001; Kimura et al., 2000; Tanaka et al., 2005), with one acting as an acid catalyst/base and the other as a nucleophilic residue (Davies and Henrissat, 1995; Sapag et al., 2002). Additionally, XYL1 has an Asp in position 55 that may be necessary for maintaining the optimum pH of these enzymes (Ellouze et al., 2011; Lübeck et al., 1997b).

Analysis of *xyl1* expression and production of endoxylanase activity

When used as the principal carbon source, glucose sustained growth of both fungal races but the maximum growth of race 0 (501.67 mg after 10 days) was delayed by approximately two days and was 29% lower when compared with race 1472 (708 mg after 8 days) (*P*<0.0001) (Figure 4A). Extracellular endoxylanase

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                                     5'UTR tacttatcattcgtccgcttcaacc
ATG GTC TCT TTC ACC CAC ATT GTC CTG GCA CTC GCG GCT TCC GCT GGA
 M   V   S   F   T   H   I   V   L   A   L   A   A   S   A   G
GTC ATC GCC AGC CCC ACT GGT GAA CTC ATC GAG AAA CGC CAG TCT ACT
 V   I   A   S   P   T   G   E   L   I   E   K   R   Q   S   T
CCA AGT TCA ACC GGC TTC CAC AAC GGC TAC TAC TAC TCG TGG TGG ACC
 P   S   S   T   G   F   H   N   G   Y   Y   Y   S   W   W   T
GAC GGT GGC TCT CAG GTC ACC TAC ACG AAC GGT GCT GGA GGC TCG TAT
 D   G   G   S   Q   V   T   Y   T   N   G   A   G   G   S   Y
AGT GTC AAC TGG GGC GGC GGC GGC GGC AAC TTT GTC GGA GGC AAG GGC
 S   V   N   W   G   G   G   G   G   N   F   V   G   G   K   G
TGG AAT CCC GGC GGT GCC AAG ACG ATC AAC TAC TCT GGA ACC TAT AAC
 W   N   P   G   G   A   K   T   I   N   Y   S   G   T   Y   N
CCG AAT GGC AAT AGT TAC CTT GCT GTT TAC GGC TGG ACA CAG AAC CCC
 P   N   G   N   S   Y   L   A   V   Y   G   W   T   Q   N   P
TTG ATT GAG TAC TAC ATC GTC GAA AAC TAC GGC ACT TAC AAT CCC GCC
 L   I   E   Y   Y   I   V   E   N   Y   G   T   Y   N   P   A
TCG CAG GCC ACG AAG AAG GGC TCT GTC ACC AGC GAC GGC GGC ACT TAT
 S   Q   A   T   K   K   G   S   V   T   S   D   G   G   T   Y
GAC ATT TAC GTC AGC ACC CGC GTG AAC CAG CCC AGC ATC GAG GGA ACA
 D   I   Y   V   S   T   R   V   N   Q   P   S   I   E   G   T
CGG ACC TTC CAG CAG TAC TGG TCG ATC CGG ACT TCA AAG CGC ACA GGT
 R   T   F   Q   Q   Y   W   S   I   R   T   S   K   R   T   G
GGC ACT GTT ACC ACT GGC AAC CAC TTC GCG GCC TGG GCT AAA GTC GGA
 G   T   V   T   T   G   N   H   F   A   A   W   A   K   V   G
TTG AAC CTT GGG AAT CAC AAC TAC ATG ATT GTG GCC ACC GAG GGC TAC
 L   N   L   G   N   H   N   Y   M   I   V   A   T   E   G   Y
TTC AGC AGT GGT TCT GCC ACG ATT ACC GTC AAC ACA CCG GCC TAG
 F   S   S   G   S   A   T   I   T   V   N   T   P   A   *

gagaagggagctgtcttgctcactattccaatcctaaacgtctcagattggaactccgaggattgcacgaagccttctcgatcgacc
agatgggaatgcacaccacgccagatgtcattctcccatgtatatacttgagacatatgccactcatcaaggccaaatgaactatga
ttgaggggtaagaaatcaaatatgccttttttcttgag 3'UTR
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Figure 1. Nucleotide and deduced amino acid sequences of the *xyl1* gene of *C. lindemuthianum* race 1472. The signal peptide sequence is underlined, the catalytic residues (Glu-96 and 187) are boxed and an asterisk indicates the stop codon.

(XYL) production by the two races was very low, with values of specific activity in the range of 6.4 and 10.5 reducing sugars/min/mg protein after 2 and 12 days of growth, respectively, for the race 1472 (Figure 4A). Corresponding values for the race 0 were 2.5 and 11.83 (Figure 4A). However, even under these conditions, significant differences in XYL production were observed with an increase for race 1472, particularly during early days of growth. For both races, a peak was observed after seven days ($P<0.0001$). The *C. lindemuthianum xyl1* transcript exhibited basal levels of expression (1-fold) in both races (Figure 5A and B).

As described earlier (Hernández-Silva et al., 2007), both races utilized xylan as a carbon substrate, but growth of race 1472 (416.67 mg of mycelia after 8 days) was over 5.5-fold higher and substantially faster than that of race 0 (75 mg of mycelia after 8 days) ($P<0.0001$) (Figure 4B). Values of 416.67 and 75 mg represent 59 and 15% of the maxima obtained in glucose-supplemented cultures, respectively. Xylan stimulated the production of XYL activity, which was increased and peaked at 90 µg reducing sugars/min/mg protein after seven days of growth and was faster in the pathogenic race ($P<0.0001$) (Figure 4B). This result was by far the highest activity detected in this study and was 1.5-fold higher than that of race 0, which reached a maximum of 59.67 µg reducing sugars/min/mg protein one day later (Figure 4B). The pathogenic race strongly expressed the *xyl1* transcript after 6 to 48 h and three days (16.5-fold) and decreased over the following 4-9 days (Figure 5A). In contrast, the non-pathogenic race weakly expressed the *xyl1* transcript between 0 h and three days, increased

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                                          5`UTR acttatcatcgtccgcttcaacc
ATG GTC TCT TTC ACC CAC ATT GTC CTG GCA CTC GCG GCT TCC GCT GGA
 M   V   S   F   T   H   I   V   L   A   L   A   A   S   A   G
GTC ATC GCC AGC CCC ACT GGT GAA CTC ATC GAG AAA CGC CAG TCT ACT
 V   I   A   S   P   T   G   E   L   I   E   K   R   Q   S   T
CCA AGT TCA ACC GGC TTC CAC AAC GGC TAC TAC TAC TCG TGG TGG ACC
 P   S   S   T   G   F   H   N   G   Y   Y   Y   S   W   W   T
GAC GGT GGC TCT CAG GTC ACC TAC ACG AAC GGT GCT GGA GGC TCG TAT
 D   G   G   S   Q   V   T   Y   T   N   G   A   G   G   S   Y
AGT GTC AAC TGG GGC GGC GGC GGC GGC AAC TTT GTC GGA GGC AAG GGC
 S   V   N   W   G   G   G   G   G   N   F   V   G   G   K   G
TGG AAT CCC GGC GGT GCC AAG ACG ATC AAC TAC TCT GGA ACC TAT AAC
 W   N   P   G   G   A   K   T   I   N   Y   S   G   T   Y   N
CCG AAT GGC AAT AGT TAC CTT GCT GTT TAC GGC TGG ACA CAG AAC CCC
 P   N   G   N   S   Y   L   A   V   Y   G   W   T   Q   N   P
TTG ATT GAG TAC TAC ATC GTC GAA AAC TAC GGC ACT TAC AAT CCC GCC
 L   I   E   Y   Y   I   V   E   N   Y   G   T   Y   N   P   A
TCG CAG GCC ACG AAG AAG GGC TCT GTC ACC AGC GAC GGC GGC ACT TAT
 S   Q   A   T   K   K   G   S   V   T   S   D   G   G   T   Y
GAC ATT TAC GTC AGC ACC CGC GTG AAC CAG CCC AGC ATC GAG GGA ACA
 D   I   Y   V   S   T   R   V   N   Q   P   S   I   E   G   T
CGG ACC TTC CAG CAG TAC TGG TCG ATC CGG ACT TCA AAG CGC ACA GGT
 R   T   F   Q   Q   Y   W   S   I   R   T   S   K   R   T   G
GGC ACT GTT ACC ACT GGC AAC CAC TTC GCG GCC TGG GCT AAA GTC GGA
 G   T   V   T   T   G   N   H   F   A   A   W   A   K   V   G
TTG AAC CTT GGG AAT CAC AAC TAC ATG ATT GTG GCC ACC GAG GGC TAC
 L   N   L   G   N   H   N   Y   M   I   V   A   T   E   G   Y
TTC AGC AGT GGT TCT GCC ACG ATT ACC GTC AAC ACA CCG GCC TAG
 F   S   S   G   S   A   T   I   T   V   N   T   P   A   *

gagaagtggagctgtcttgctcactattccaatcctaaacgtctcagattggaactccg 3´UTR
```
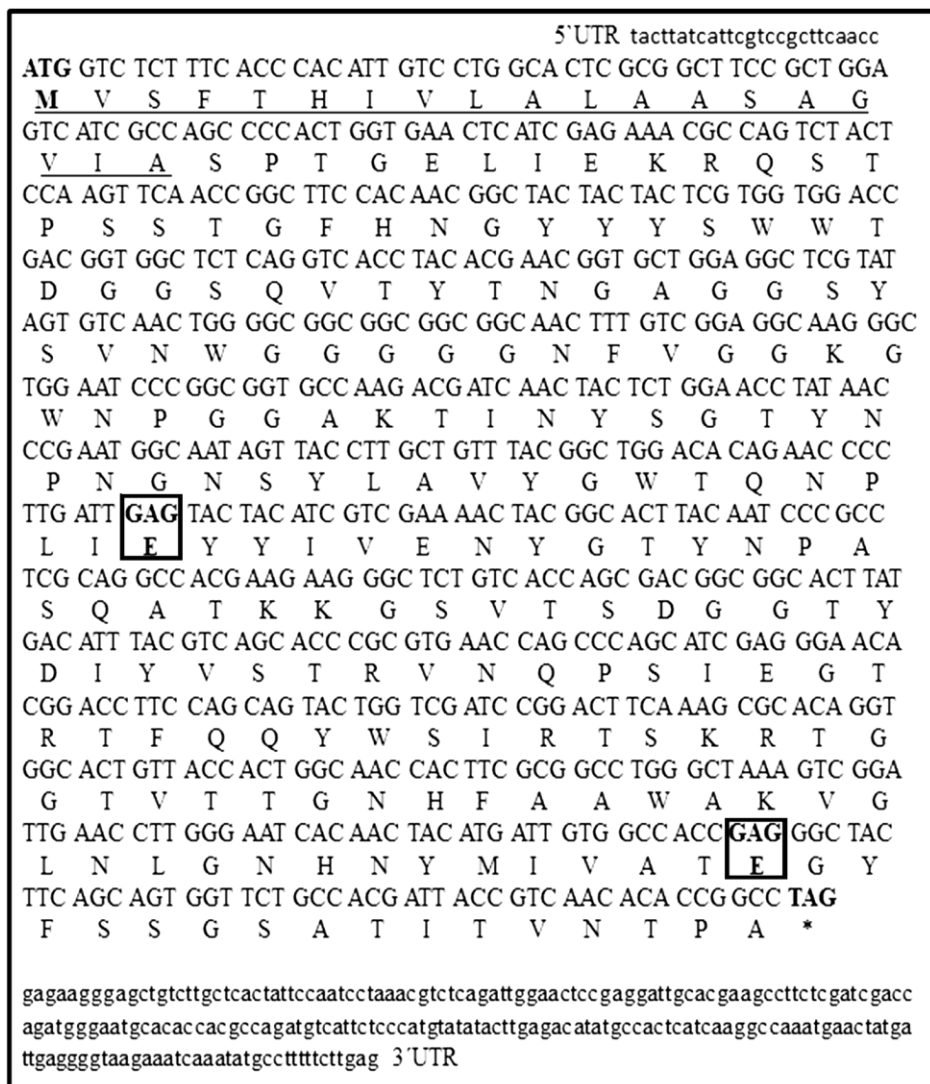
Figure 2. Nucleotide and deduced amino acid sequences of the *xyl1* gene of *C. lindemuthianum* race 0. The signal peptide sequence is underlined, and the catalytic residues (Glu-96 and 187) are boxed and an asterisk indicates the stop codon.

and peaked after four days (6.6-fold) and decreased over the following 5-9 days (Figure 5A).

The ability of cell walls fractions from *P. vulgaris* to sustain the growth of *C. lindemuthianum* and induce endoxylanase was also tested. As observed with other carbon sources, the pathogenic race grew faster and to a greater extent than the non-pathogenic race. This difference was maintained up to approximately 8-9 days of incubation (*P*<0.0001) (Figure 4C). After that, both races grew with similar rates producing comparable amounts of mycelium protein after 12 days of growth (*P*>0.05). Due to technical restrictions to measure growth on cell walls, values obtained with this substrate could not be compared with those obtained in soluble carbon substrates. The pathogenic race exhibited low expression levels of the *xyl1* transcript during early time points, then

a weak peak was observed after 48 h (3.3-fold), and this increased over the following 5-9 days (9.3-fold) (Figure 5B). In contrast, the non-pathogenic race weakly expressed the *xyl1* transcript between 0-48 h, then it increased, peaked after 3 and five days (~2-fold) and finally decreased over the following 7-9 days (Figure 5B). Interestingly, after eight days of incubation, the pathogenic race produced an amount of XYL activity (80 µg reducing sugars/min/mg protein) equivalent to 89% of the maximum observed after seven days of growth on xylan (Figure 4C and B). The non-pathogenic race produced a 1.6-fold lower amount of enzymatic activity(50 µg reducing sugars/min/mg protein) that corresponded to 67% of the maximum induced by xylan (Figure 4C and B). It should be noted that in this case, the maximum XYL activity was produced by the
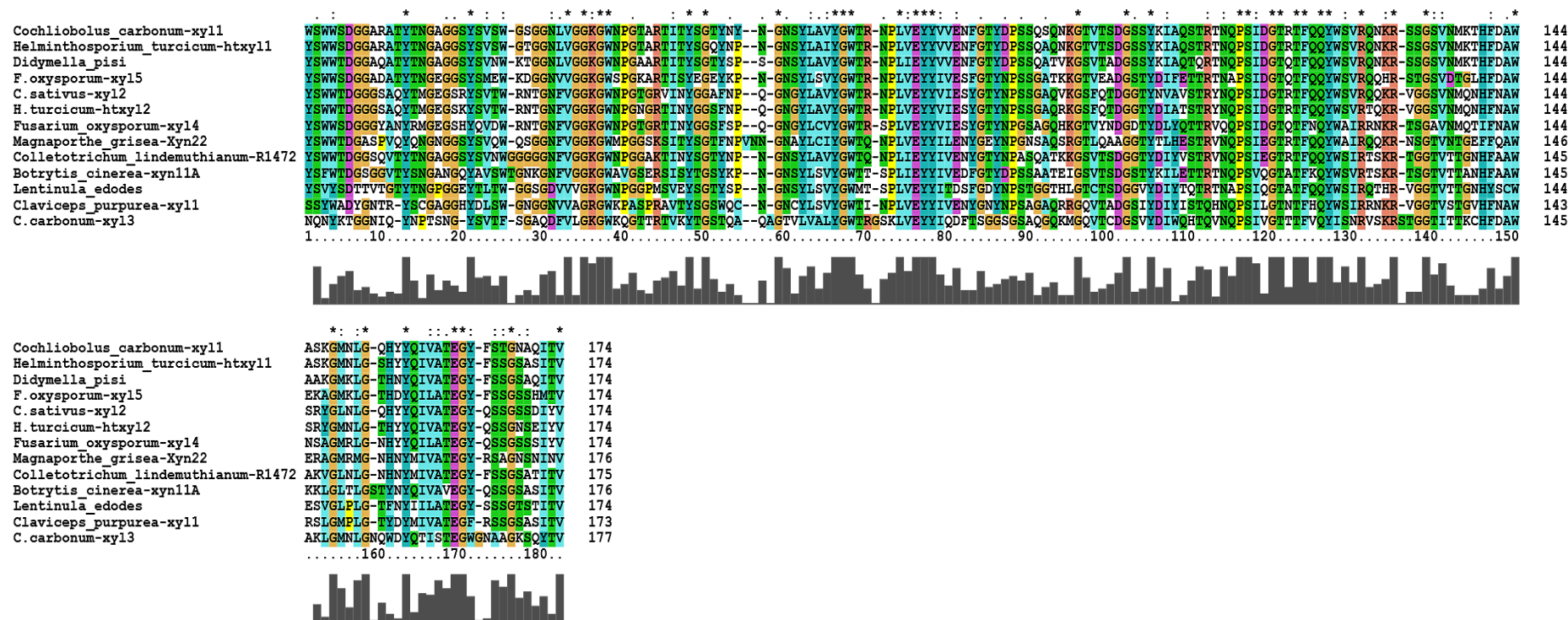
Figure 3. Clustal alignment of endoxylanases. Identical residues and conserved catalytic residues are marked with an asterisk (*). Dots indicate the change of one amino acid for another of the same group.

non-pathogenic race two days after the maximum produced by the pathogenic race.

The regulation of lytic enzymes in some fungi (St. Leger et al., 1988) and gene expression in several species of *Colletotrichum* can be modulated by ambient pH (Prusky et al., 2001) and this mechanism of regulation operates in a vast diversity of organisms (Denison, 2000). Therefore, we considered it necessary to determine whether the pH of the extracellular medium varied during the period of incubation under the different culture conditions. Throughout

this study, the initial pH of all culture media was adjusted to 5.5. The growth of the pathogenic race in the presence of glucose, xylan or cell walls resulted in a rather irregular profile of pH variation. After 12 days, the pH reached values of 6.0, 6.5 and 7.2, respectively (Table 2). On cell walls, an abrupt acidification of the medium was observed after two days and then it became increasingly alkaline. The growth of the non-pathogenic race on glucose or xylan resulted in a steadier pattern of pH alkalinization to final corresponding values of 6.2 and 6.5. Alkalinization of the medium to pH

7.0 by race 0 grown on plant cell walls followed a more irregular profile.

Phylogenetic analyses

Comparison of amino acid sequences of endoxylanases showed 40 to 67% identity suggesting a diversification process that gave rise to proteins that shared identity mainly in sequence and the structure of the catalytic site. Clustal alignment identified the location of amino acids

A

B

C
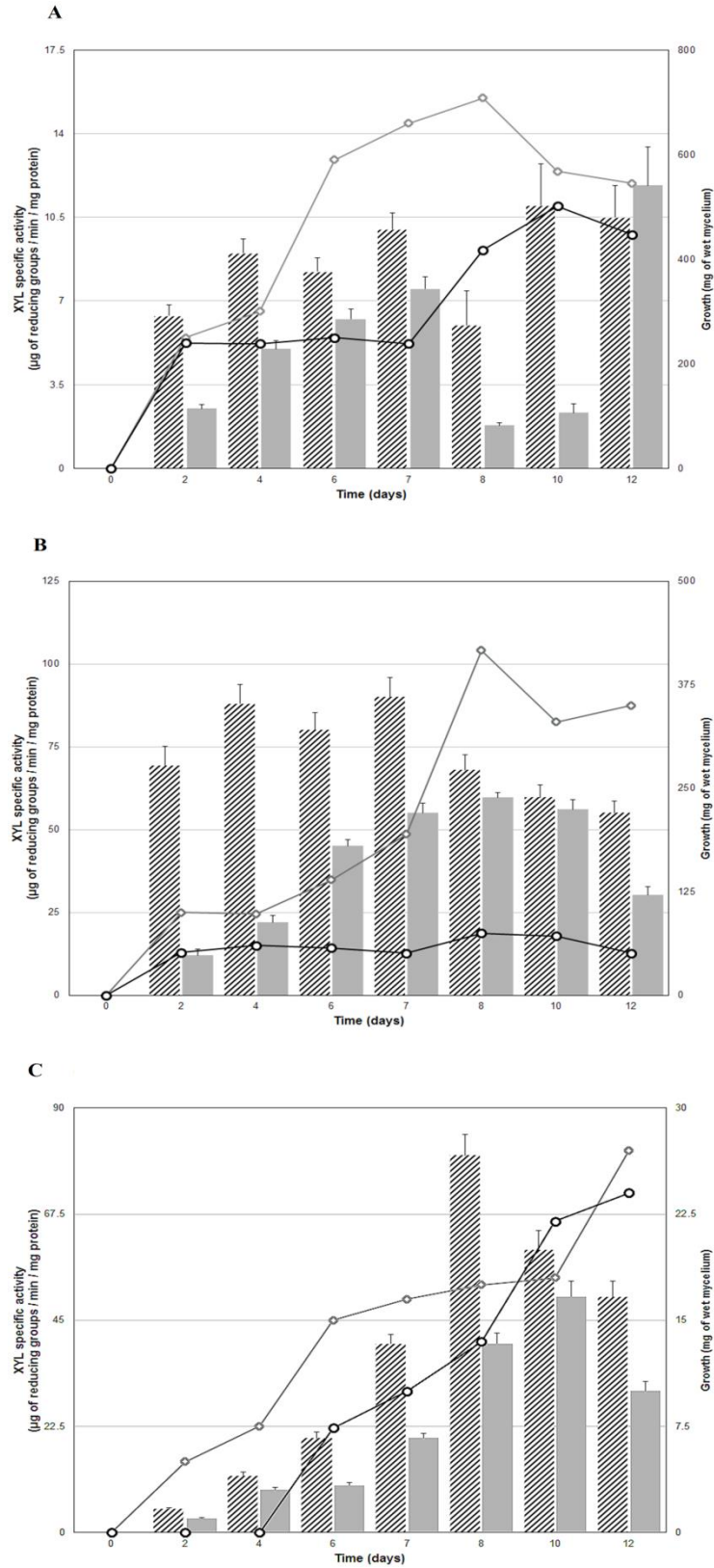
Figure 4. Growth and production of extracellular endoxylanase activity (XYL) by race 1472 and race 0 cultivated in the presence of glucose (A), xylan (B), or plant cell walls (C) as the sole carbon sources. Diamonds, the growth of race 1472; circles, the growth of race 0; stripped bars, XYL activity of race 1472; gray bars, XYL activity of race 0.

A

B
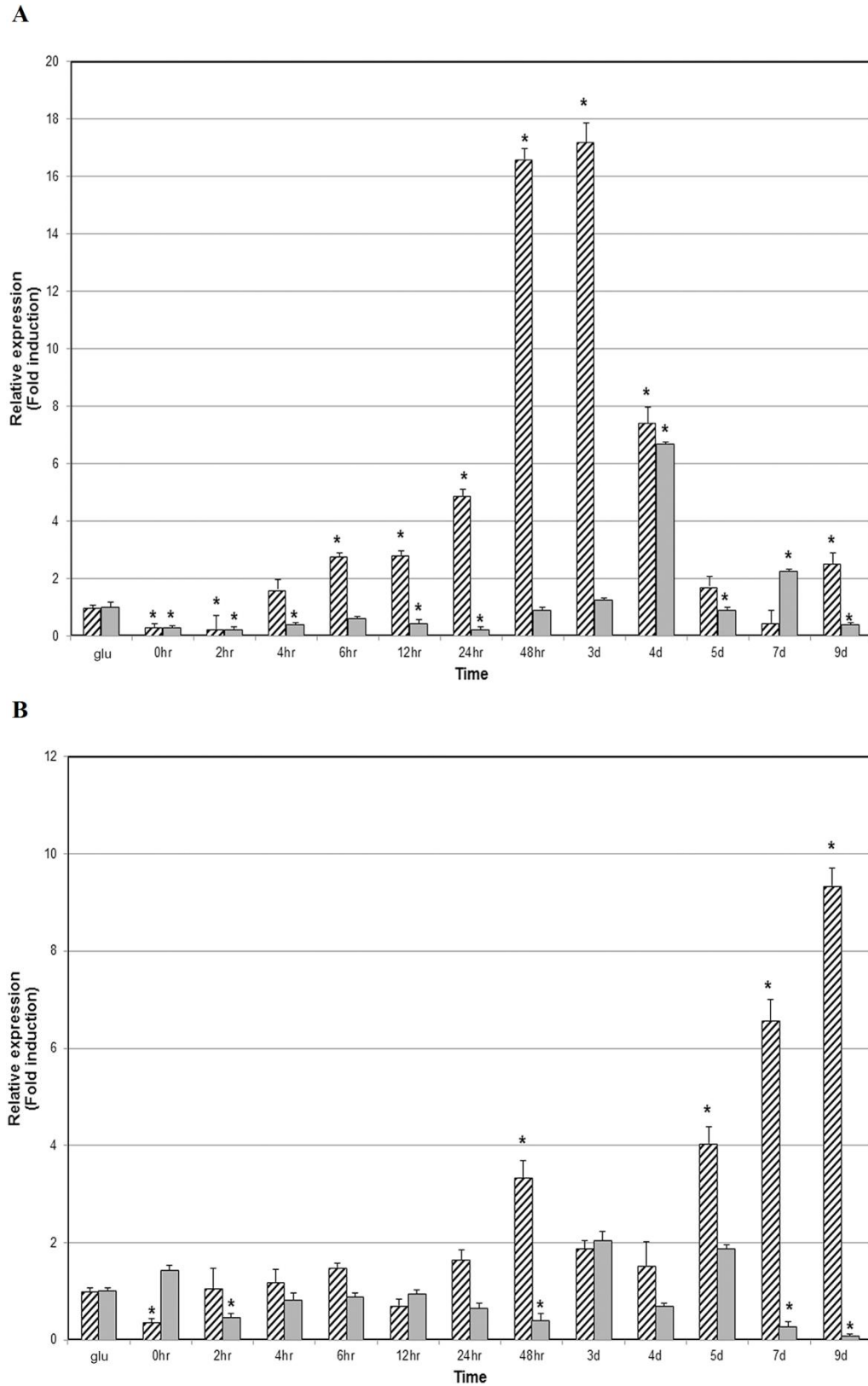
Figure 5. Expression analysis of *xyl1* by RT-qPCR induced with xylan (A) or bean cell walls (B). Stripped bars, show *xyl1* expression in race 1472; gray bars, show *xyl1* expression in race 0. Each bar indicates the mean of triplicates ± SE of three independent experiments. The symbol "*" indicates significant changes (*P*<0.05) about the control (glu, glucose).

Table 2. Kinetic data of pH analysis in races 1472 and 0 of *C. lindemuthianum,* grown with 2.5% of glucose, xylan or *P. vulgaris* cell walls.

Time*	Race 1472			Race 0		
	Glucose	Xylan	Cell walls	Glucose	Xylan	Cell walls
0	5.5	5.5	5.5	5.5	5.5	5.5
2	5.66	5.585	4.43	5.52	5.605	5.5
4	6.79	6.04	6.5	5.63	6.015	6.08
6	5.6	6.88	6.93	5.81	6.09	7.06
7	5.92	6.485	6.91	5.93	6.295	6.36
8	4.83	7.215	7.99	6.13	6.655	6.66
10	4.95	6.705	8.05	6.39	6.79	7.21
12	6.03	6.66	7.41	6.18	6.725	7.15

*Time expressed in days.
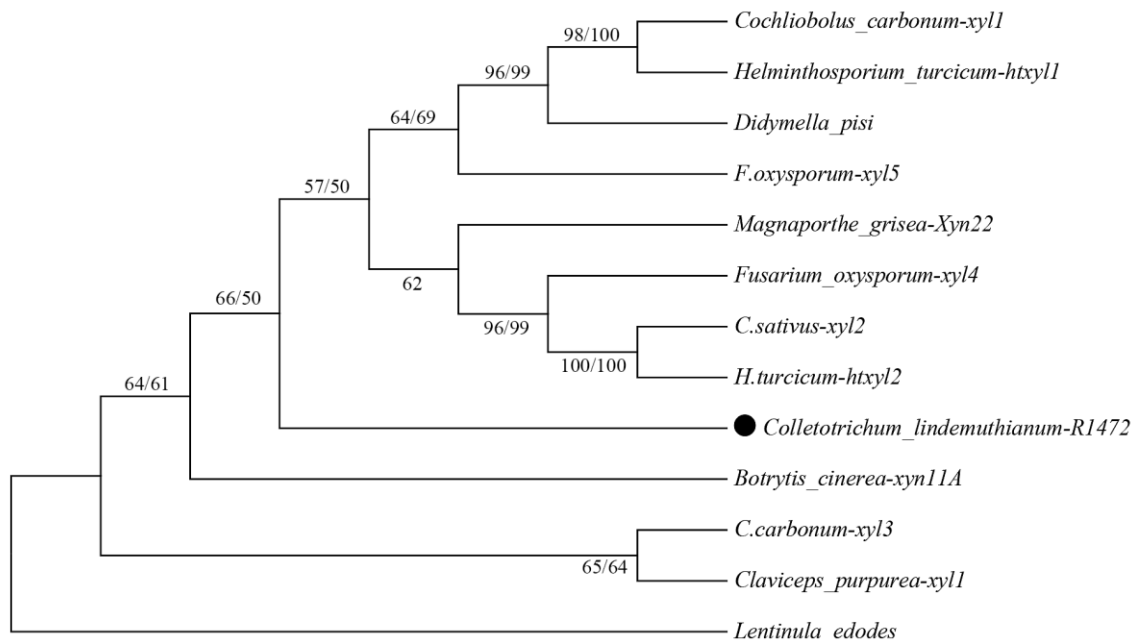
Figure 6. Phylogenetic analyses of endoxylanases from *C. lindemuthianum* and other fungal species. The tree was constructed using the Neighbor-Joining (NJ) and *Maximum* likelihood methods and includes only the conserved region of the catalytic domain (190 aa) of the proteins used in the analysis. General topology obtained is represented by the 50% of majority rule consensus tree, in which the NJ posterior probabilities and ML bootstrap support are indicated on the branches. The numbers at the nodes indicate bootstrap values based on 1,000 bootstrap replications.

in endoxylanases expected to have a catalytic role (Figure 3) (Ellouze et al., 2011; Sapag et al., 2002). The phylogenetic analyses revealed xyl3 of *Cochliobolus carbonum* and xyl1 of *Claviceps purpurea* in a basal clade (Figure 6). Next, xyn11A of *Botrytis cinerea* was separated as the next version of these enzymes. Later, in an evolutionary progression, the rest of enzymes were grouped into a widely diversified clade with XYL1 of *C. lindemuthianum* as basal of two sub-clades or lineages. In one of these sub-clades, other protein (xyl1) of *C. carbonum* grouped with htxyl1 of *H. turcicum*, the enzyme

of *Didymella pisi* and xyl5 of *Fusarium oxysporum,* was found. In the other sub-clade, htxyl2 of *Helminthosporium turcicum* grouped with xyl2 of *Cochliobolus sativus*, xyl4 of *Fusarium oxysporum* and xyn22 of *Magnaporthe grisea* were found (Figure 6).

DISCUSSION

No differences were found in the coding region of the endo-β-1,4-xylanase of the non-pathogen and pathogen

races of *C. lindemuthianum.* The *xyl1* sequence analysis and Clustal alignment with xylanases reported for other fungi revealed the characteristic elements of genes coding for endo-β-1,4-xylanases of family GH11.

Induction of cell wall degrading enzymes by different carbon substrates has been studied in some phytopathogenic fungi such as *Sclerotinia sclerotiorum* (Riou et al., 1991), *Sclerotium rolsfii* (Sachslehner et al., 1998) and *Penicillium* sp. (Rahman et al., 2003), among others. The involvement of some of these enzymes in the development of bean anthracnose by *C. lindemuthianum* race ɣ was first described by Wijesundera et al. (1989). Results presented here show a clear difference between the non-pathogenic (0) and pathogenic (1472) races of *C. lindemuthianum* regarding growth, induction of *xyl1* transcript expression and production of extracellular endoxylanase activity when they are challenged with different carbon substrates. Accordingly, though maximum growth was obtained on glucose, a readily metabolizable nutrient, basal expression of *xyl1* and only trace amounts of XYL were produced by both races; however, although low, a higher XYL production was observed for race 1472, particularly early during incubation. A similar basal production of xylanase and endoglucanase (Sachslehner et al., 1998; Tuncer et al., 2004), pectinases (Hernández-Silva et al., 2007; Oyeleke et al., 2012; Riou et al., 1991) and cellulases (Acosta-Rodriguez et al., 2005; Carle-Urioste et al., 1997; Sharada et al., 2013) has been described in the fungi *S. rolsfii*, *S. sclerotiorum* and *C. lindemuthianum*, respectively.

In fungi, the expression of extracellular hydrolytic enzymes is coordinately regulated by transcriptional activators and repressors (Aro et al., 2005; Tani et al., 2014). The expression of genes encoding xylanolytic enzymes is subject to catabolic repression through the action of CreA under a preferred carbon source and the activation through the action of XlnR under carbon limitation (Cho and Choi, 1999; de Vries and Visser, 2001; Tani et al., 2014).

In this study, significant levels of the *xyl1* expression and endoxylanase were produced in pathogenic race 1472 only when the enzyme substrate was available in a medium lacking other nutrients of easier assimilation, such as glucose, indicating that expression of *xyl1* can be regulated by the carbon source. It has been proposed that basal levels of endoxylanase commence degradation of xylan generating products that induce further enzymatic activity. Xylose, as the final product of xylan degradation, functions as a regulator of the expression of xylanases, acting as an inducer at low concentrations and as a repressor at high levels (de Vries et al., 1999; de Vries, 2003; Kulkarni et al., 1999; Mach-Aigner et al., 2010, 2012; Stulke and Hillen, 2000). These results support this idea as degradation of xylan occurred when basal levels of endoxylanase activity produced low levels of xylose, which then induced expression of higher levels

of expression of *xyl1* and enzyme production in the pathogenic race 1472. At later time points, a reduction of activity was observed most likely due to repression by the accumulation of xylose.

The non-pathogenic race of *C. lindemuthianum* used in this work is unable to infect *P. vulgaris*, and thus its lifestyle is closer to that of a saprophytic fungus. Therefore, it is possible that the differences found between the non-pathogenic and pathogenic races of *C. lindemuthianum* are related to the speed of activation of the lytic enzyme genes during the interaction with the host. Additionally, the pathogenic race degrades xylan faster and grows better than the non-pathogenic race, suggesting a different ability in the degradation of this polysaccharide and the use of oligo- or monomeric sugars. As previously described (Hernández-Silva et al., 2007; Lara-Marquez et al., 2011), the expression of *Clpnl2* gene and activity of pectin lyase between the two races were similar to that observed in this study when 92% esterified pectin was utilized as the sole carbon source. In other words, the pathogen requires a rapid and higher level expression of endoxylanase activity and other related lyticases for successful interaction with the live plant tissue, which implies an energy cost and the non-pathogen does cannot invest because it feeds on dead plant tissue.

The response of the fungus to cell walls from *P. vulgaris* is interesting if it is considered that only a certain proportion of the provided substrate corresponds to xylan. A typical primary cell wall contains 9-25% cellulose, 25-50% hemicellulose (whose main structural polymer is xylan), 10-35% pectin and 10% proteins (Cosgrove, 1997). The authors previously demonstrated that *P. vulgaris* cell walls also induced pectin lyase (PNL) activity in the pathogenic race to levels that represent up to 46% of the maximum obtained with 92% esterified pectin, the best PNL inducer. This value was approximately 2.8-fold higher than that produced by race 0 (Hernández-Silva et al., 2007). This difference is close to that observed for endoxylanase activity in this study. These data indicate that polysaccharides present in the cell wall structure cooperate efficiently to induce a range of polysaccharidases specific for the types of glycosidic linkages present in the cell wall components, thus granting the fungus the ability to degrade the wall barrier efficiently.

A number of evidences indicate a role of ambient pH in the regulation of production of pectinolytic enzymes in fungi such as *Penicillium paxilli* (Szajer and Czajer, 1985), *P. itallicum* (Alaña et al., 1989), the avocado-pathogen *C. gloeosporioides* (Drori et al., 2003; Kramer-Haimovich et al., 2006; Yakoby et al., 2000), *Trametes trogii* (Levin and Forchiassin, 1998) and *Aspergillus oryzae* (Fontana and Silveira, 2012). In the latter, alkalinization during fruit infection is necessary for the conversion of the biotrophic stage into the necrotrophic stage (Kramer-Haimovich et al., 2006). Also, ambient pH

has been described as a regulatory factor related to the pathogenesis of *S. sclerotiorum* (Rollins and Dickman, 2001), *C. gloesporoides* (Alkan et al., 2013) and *C. acutatum* (You et al., 2007). Contrary to these findings, an effect of the pH on growth, expression of *xyl1* gene and production of XYL activity by *C. lindemuthianum* was not observed, which is consistent with previous results on PNL activity (Hernández-Silva et al., 2007).

The results, allow us to hypothesize that the regulation of enzyme expression and nature of the assimilatory carbon substrates processed by these organisms play a determinant role in their lifestyle. The differences in growth, *xyl1* expression and production of enzymatic activity between the two races of *C. lindemuthianum* suggest an adaptation of race 1472 that results in a rapid degradation of xylan, induction of increased activity and utilization of depolymerization products as carbon nutrients. Race 0 does not seem to prefer xylan as a carbon source but instead grows better with bean cell walls, suggesting that differences exist in the utilization of mono- or oligosaccharides on race 1472. The authors observed a similar behavior of other enzymes of the complex involved in the degradation of the cell wall suggesting that it may be a general phenomenon (Acosta-Rodriguez et al., 2005; Hernández-Silva et al., 2007; Lara-Marquez et al., 2011). The differences at this level can be part of the general response of fungi to host components. However, future studies comparing the enzymatic complexes of degradation of more fungal species with different lifestyles will be required to confirm this hypothesis.

Finally, phylogenetic analyses showed a diversification of endo-β-1,4-xylanases and separation of proteins from the same fungal species into various groups or lineages. Similar results were described after a phylogenetic analysis of the nucleotide sequences of the *htxyl1* and *htxl2* xylanase genes from the corn pathogen, *H. turcicum.* These genes showed differential expression related to the substrate type (xylan and/or xylose) or stages of infection of maize, suggesting a role in saprophytic or pathogenic phases (Degefu et al., 2004; Ellouze et al., 2011). Here, a phylogenetic separation of other xylanases with differential expression was found; *xyl1* and *xyl3* in *C. carbonum* (Apel-Birkhold and Walton, 1996) and *xyl4* and *xyl5* in *F. oxysporum* (Gomez-Gomez et al., 2001, 2002), suggesting diversifying selection (Brunner et al., 2013). In this context, the differential expression of cutinases, cellulases, hemicellulases and pectinases related to different stages of the life cycle, namely, biotrophic, necrotrophic and saprophytic, has been reported in the hemibiotrophic pathogen *Zymoseptoria tritici* (Brunner et al., 2013). Also, purified selection has been detected in many genes, which can be related to the optimization of enzymatic activity. In some of these genes, diversifying selection has also been detected, which is possibly related to the adaptation to the host and/or the life cycle of the fungus (Brunner et

al., 2013).

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Full Length Research Paper

Effects of temperature and incubation time on growth and ochratoxin A biosynthesis by *Aspergillus carbonarius* and *Aspergillus ochraceus* in grain-based media

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Ochratoxin A (OTA) has been frequently found as a food contaminant, and it is considered one of the mycotoxins most harmful to human health. In this context, this study was performed aiming to evaluate mycelial growth and ochratoxin A production from three isolates of *Aspergillus carbonarius* and *Aspergillus ochraceus*. The isolates were inoculated on culture media coffee, wheat and maize as well as YES medium and cultivated at 15 and 25°C. Measurement of colony diameters was performed every 48 h for growth description. OTA quantification was made with high-performance liquid chromatography (HPLC) three times (5th, 15th and 25th day). All the isolates presented higher growth rate in YES medium at 25°C; however, growth in every culture media at both tested temperatures was observed. The maize-based medium showed the lowest growth rate. YES medium induced the most OTA production. In grain-based culture media, *A. ochraceus* isolates produced lower quantities of OTA, reaching a maximum of 9.95 μg/g. However, *A. carbonarius* isolates produced much higher quantities, showing the *A. carbonarius* CCDCA10608 isolate, which produced 29.83 μg/g at 15°C in YES medium and 13.06 in wheat-based medium at 15°C. Therefore, among the tested conditions, those more and less favorable to OTA production were recognized.

Key words: *Aspergillus*, food, high performance liquid chromatography, mycotoxin, ochratoxin A.

INTRODUCTION

Ochratoxin A (OTA) is a mycotoxin with a high nephrotoxicity potential, besides showing hepatotoxicity, teratogenicity, carcinogenicity, cytotoxicity, neurotoxicity and immunosuppressive properties (Woo et al., 2012; Stachurska et al., 2013; Solcan et al., 2013; von Tobel et al., 2014; Gayathri et al., 2015; Calado et al., 2015). They

are detected in different foods such as coffee (Batista et al., 2009; Casal et al., 2014), grain, like wheat and maize and their derivatives (Juan et al., 2008; Gang et al., 2014; Al-Hadithi et al., 2015), wine (Terra et al., 2013; Giovannoli et al., 2014), powdered guarana (Martin et al., 2014), among others (Obanos et al., 2005; Vidal et al., 2015, Lippolis et al., 2014; Pacheco et al., 2015; Marino et al., 2014). At first, it was believed that OTA was produced only by *Aspergillus ochraceus* and *circumdati-*related species, as well as *Penicillium verrucosum*, though these species are not considered the main OTA sources in some food because OTA may also be produced naturally by *Aspergillus carbonarius* (Van der Merwe et al., 1965; Horie, 1995, Lund and Frisvad, 2003; Schabo et al., 2015; Kogkaki et al., 2015), since 75-100% of the isolates were found to be ochratoxigenic (Romero et al., 2005). Fungal growth and consequent OTA production are determined by a wide range of parameters, classified as physical, chemical and biological, as well as by interactions encompassing these factors (Nielsen et al., 2003). OTA biosynthesis may be influenced by eco physiological intrinsic factors, such as temperature, nitrogen shortage and pH conditions which also influence germination, growth and sporulation (Palacios-Cabrera et al., 2005; Garcia-Cela et al., 2014; Passamani et al., 2014). Under stress conditions, the signaling pathways certainly induce the production of mycotoxins (Choi et al, 2008; Kohut et al., 2009).Therefore, understanding the effects of these factors can help reduce mycotoxins, both in the field and during storage. Furthermore, interactions between mycotoxigenic species and other spoilage fungi can significantly influence ochratoxin A production (Lee and Magan, 2000).Several authors have studied the ability of *A. ochraceus* and *A. carbonarius* to produce OTA in coffee, maize and synthetic grape and barley medium under different environmental conditions (Pardo et al., 2005; Lee and Magan, 2000; Marín et al., 2006; Pardo et al., 2004; Garcia et al., 2011). Approximately, 25% of the entire annual grain production is affected by the presence of mycotoxins, leading to imminent risk of health problems associated with the ingestion of contaminated products, highlighting coffee, maize and wheat (Lawlor and Lynch, 2001). Each fungal strain has its own physiological peculiarities and hence different habitats. The water activity and temperature are very important because they affect the growth and production of OTA (Pardo et al., 2005; Kapetanakou et al., 2009).Thus, in this study, the authors aimed to investigate the necessary conditions for growth and synthesis of OTA by *A. carbonarius* and *A. ochraceus* as well as their concentration in different food-based culture media.

MATERIALS AND METHODS

Fungal strains and inoculation

Three *A. carbonarius* strains (CCDCA10610, CCDCA10609 and CCDCA10608) isolated from grape and three *A. ochraceus* strains (CCDCA10613, CCDCA10612, CCDCA10611) isolated from coffee, all ochratoxin A producers, were used in the study. They were provided by the Coleção de Cultura de microrganismos do Departamento de Ciências dos Alimentos – UFLA (CCDCA) (Culture Collection of Food Science Department – UFLA). The strains were transferred to Petri dishes containing Czapek Dox Agar (Sigma-Aldrich, St. Louis, MO) and were incubated at 25°C for 7 days. The spore suspension was prepared using 30 mL of sterile distilled water containing 0.05% Tween 80 (Sigma-Aldrich) and was then filtered through sterile gauze (Nexcare, 3M, São Paulo, Brazil). A 10 mL aliquot of the suspension was transferred to a Neubauer chamber (Sigma, São Paulo, Brazil) to determine the final spore concentration. Two microlitres of the spore concentration (10^6 spores per ml), were placed in the centre of the prepared plates and incubated at 15 and 25°C. Each strain was inoculated in three independent plates in each medium.

Culture media

The strains were cultivated on five different agar culture media. Malt extract agar (MEA) and yeast extract sucrose (YES) media were purchased from Merck (Darmstadt, Germany) and prepared according to manufacturer suggestion. Coffee, wheat and maize were prepared as follows: coffee extract agar (CMEA), 175 g ground green coffee and 20 g agar were dissolved in 1 L distilled water (Pardo et al., 2005); Wheat Extract Agar (WMEA), 175 g wheat flour, 20 g agar were dissolved in 1 L of distilled water (Muñoz et al., 2011); Maize Agar (CMA), 30 g maize flour, 20 g agar were dissolved in 1 L distilled of water (Ramos et al., 1998). Flours and 1 L of water were placed in clean fabric and boiled at low heat for 60 min. The fabric was then wrung and the liquid filtered in hydrophilized gauze and the liquid completed to 1 L. Finally, the agar was added and the resulting product was sterilized at 121°C for 15 min in an autoclave.

Assessment of strain growth and OTA extraction from cultures

Petri dishes were examined daily and the colony diameters were measured in perpendicular directions using a digital caliper on alternate days during the experiment. OTA was extracted according to the modified method of Bragulat et al. (2001). Three plugs of culture were removed from the center, middle and edge of each colony on the 5th, 15th and 25th day of the incubation period. The plugs were weighed in test tubes, and then 1 mL of methanol was added. The tubes were homogenized vigorously for 5 s and kept at 25°C for 60 min. The extracts were filtered through polytetrafluoroethylene membranes (0.22 µm; Millipore Corp., Billerica, MA) and were then analyzed using a Shimadzu high-performance liquid chromatograph coupled to two high-pressure pumps (model SPD-M20A), degasser DGU 20A$_3$, interface CBM-20A, auto injector SIL-10AF, and RF-10 A$_{xl}$ fluorescence detector (Shimadzu, Kyoto, Japan). The Zorbax Eclipse XDB-C18 column (4.6 by 250 mm, 5 µm; Agilent Technologies, Palo Alto, CA) was used, connected to a Zorbax Eclipse XDBC18 4-pack precolumn (4.6 by 12.5 mm, 5 µm; Agilent). The chromatographic conditions for wavelength were 332 nm for excitation and 476 nm for emission. The flow used was 0.8 mL min^{-1}, and the injected volume of samples and standard was 20 µL. The elution was performed using an isocratic system of 35:35:29:1 (methanol–acetonitrile–water–acetic acid). The average retention time for OTA determination was 11 ± 0.1 min. The amount of OTA in the samples was determined using an analytical curve obtained by linear regression (y = 1.11756 X $10^7 x$ - 2,592.1485, where y is the peak area and x is the OTA concentration). The calculation defined the peak area versus the concentration of the respective standard solution, obtained by

Figure 1. Growth of all *Aspergillus* isolates (6) at 15°C in different culture media: a) YES; b) CMEA; c) WMEA; d) CMA.

setting the coefficient of determination (R^2) at 0.9999. The detection limit (DL) and quantification limit (QL) were estimated through parameters obtained by the analytical curve and were calculated according to the following: DL=3 SD/m and QL=10 SD/m (where SD is the standard deviation and m is the angular coefficient of the linear regression) (Harris, 2008). The values obtained for DL and QL were 0.0004 and 0.0016 μg/g, respectively. All the samples were analyzed in duplicate, and the standard OTA solutions were assessed in triplicate.

Recovery assays

Recovery assays were performed to ensure the analytical quality of the results. The semisynthetic culture medium was fortified with concentrations equal to 1.0, 3.0, and 6.0 μg/g in triplicate. The samples were extracted with methanol and analyzed according to the method of Bragulat et al. (2001).

The results of the recovery assays used were 82, 87, and 91%, respectively. These recoveries proved the remarkable reproducibility of the method and complied with Codex Alimentarius requirements for analytical methods (70 to 110% recovery) (Codex, 2008).

Statistical analysis

Statistical analysIs for the determination of OTA were obtained with SISVAR computer software developed by Ferreira (2000), considering the split-plots as each investigated individual per species. Data from OTA production were analyzed considering time (5[th], 15[th] and 25[th] days), culture medium (YES, CMEA, WMEA and CMA) and temperature (15 and 25°C). Treatment means were compared by the Scott Knott test (1974) at 5% probability.

RESULTS

Mycelial growth

The growth of *A. carbonarius* and *A. ochraceus* isolates cultivated in different culture media, temperature and time is shown in Figures 1 and 2. All the isolates had similar growth in YES at 15°C, reaching a maximum on the plate on around the 18[th] day. The same medium at 25°C temperature also showed maximum growth for all the
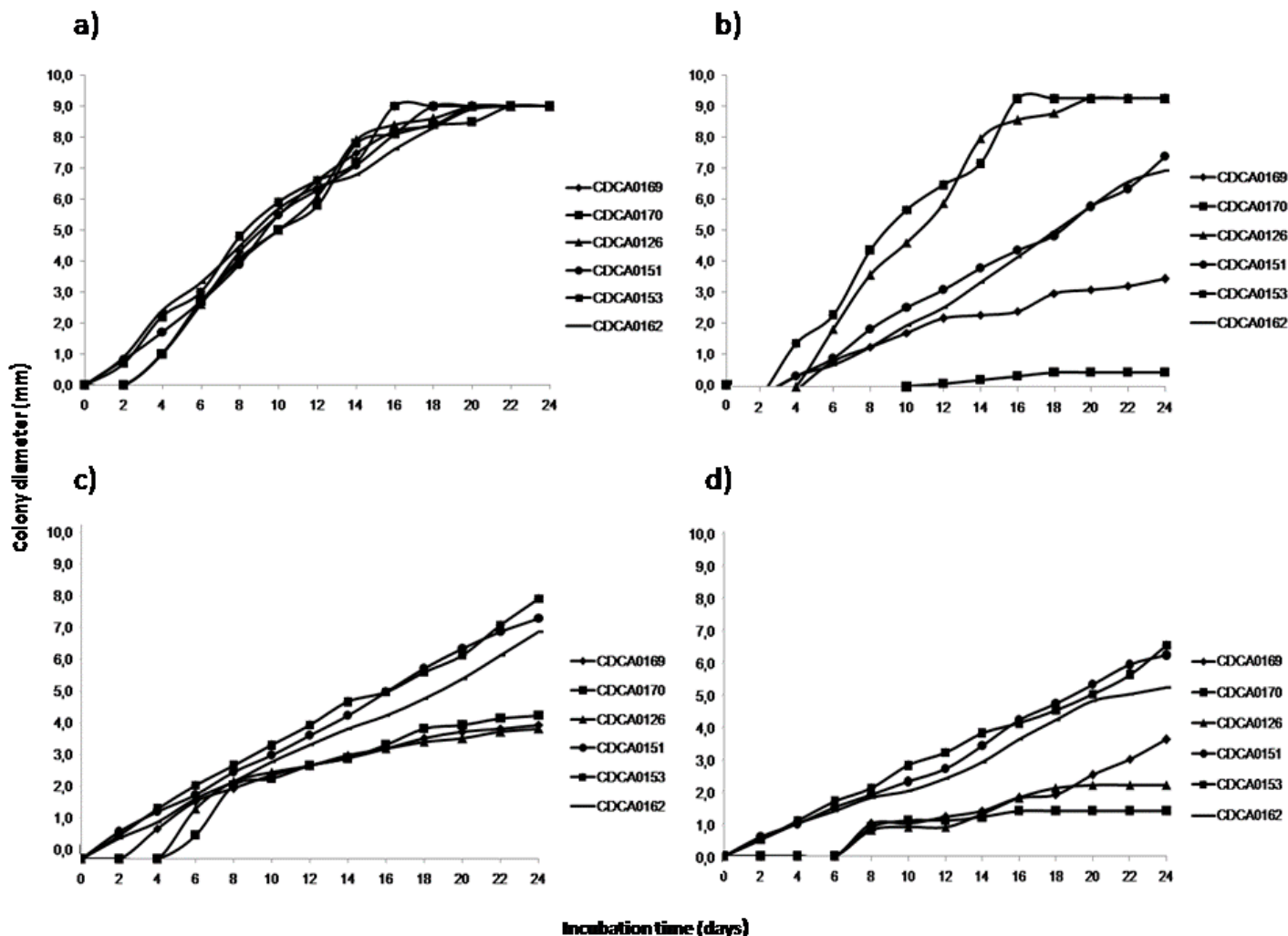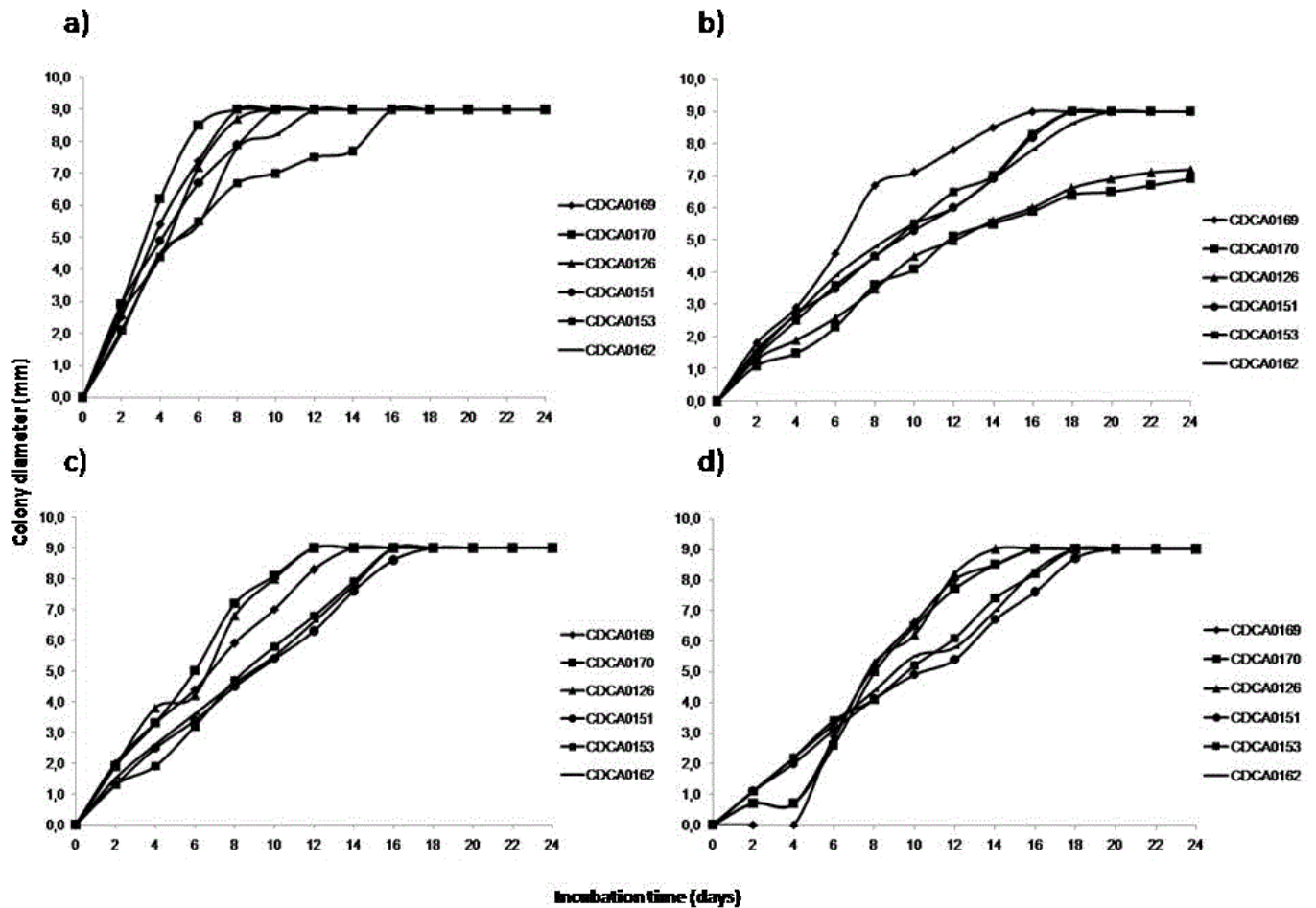
Figure 2. Growth of all *Aspergillus* isolates (6) at 25°C in different culture media: a) YES; b) CMEA; c) WMEA; d) CMA.

isolates, but this index was achieved at about 10 days.

On the coffee extract medium, the growth varied. *A. carbonarius* CCDCA10610 and *A. ochraceus* CCDCA10612 isolates had the highest growth rates for the 15°C temperature treatment with a maximum growth around the 17[th] day. *A. carbonarius* CCDCA10609, however, had a very slow growth and it did not exceed 2 cm diameter at the end of 24 days. In the treatments at 25°C temperature, *A. carbonarius* CCDCA10609 isolate also had a lower growth rate in comparison with the other fungi, attaining a maximum growth of 7 cm diameter.

The growth of isolates in maize and wheat based media at 15°C temperature were similar. *A. ochraceus* isolates grew relatively faster in comparison with *A. carbonarius* isolates. However, no isolate growth reached throughout the whole. *A. carbonarius* isolates achieved growth on the whole plate on the 11[th] day in wheat medium at 25°C, and *A. ochraceus* isolates on the 16[th] day. All isolates had maximum growth between the 15[th] and 18[th] day in maize medium at 25°C. The results reveal that the growth at 15°C was generally slower, regardless

of medium and isolate. However, YES medium was markedly more favorable to fungal development at both temperatures.

A. carbonarius presented traditional morphological characteristics, such as white mycelium and black spores, even though each medium interfered in some colony features. *A. ochraceus* also displayed their common features, such as yellowish spores, although variations in each culture media have also been observed.

Ochratoxin A production

OTA concentration values ranged from <QL - 30.42 μg/g. YES culture media stood out as the best substrate for OTA production in all treatments (Figure 3). Tables 1 and 2 show the mean OTA concentration obtained *in vitro* by isolates belonging to *A. ochraceus* and *A. carbonarius*, respectively, in relation to culture media, temperature and incubation time. *A. carbonarius* produced a greater amount of OTA in comparison with *A. ochraceus,* the *A.*

Figure 3. HPLC chromatograms of treatments in different factor combinations. (A) *A. carbonarius* CCDCA10608 in YES medium on the 15th day at 15°C; (B) *A. ochraceus* CCDCA10611 in YES medium on the 25th day at 25°C.

Table 1. Ochratoxin A concentration produced by three *A. ochraceus* isolates in different culture media, temperature and incubation time.

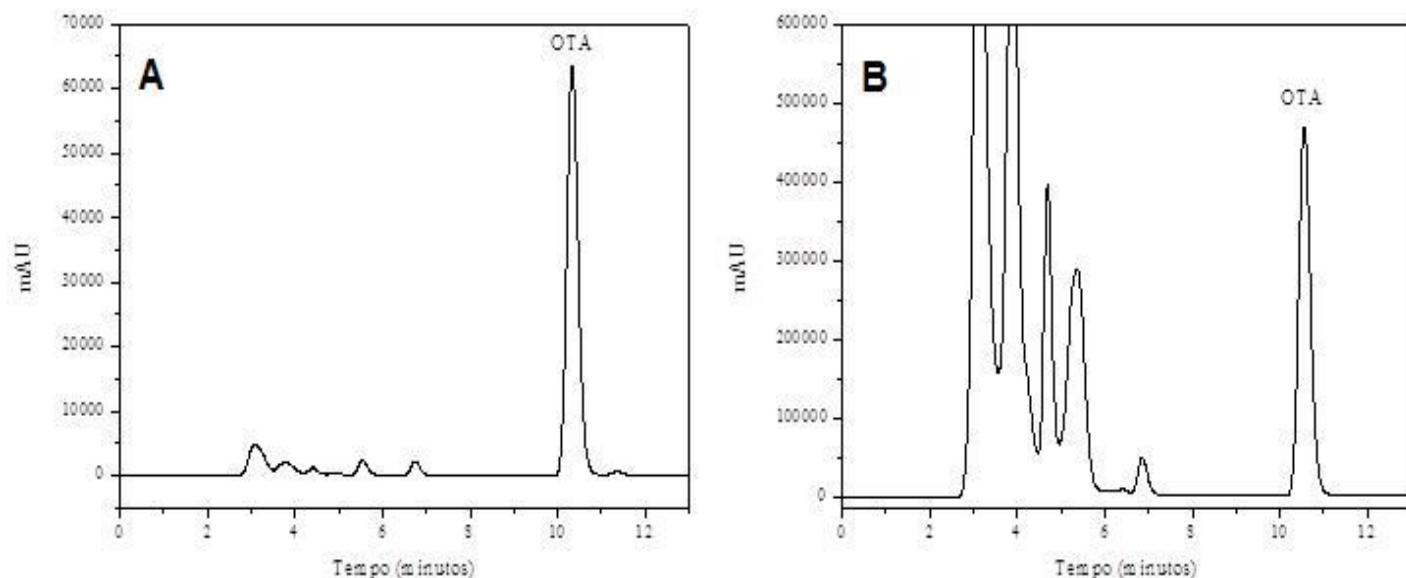| Strain	Medium	OTA concentration (ug g^{-1})* ± SD					
		15ºC			25ºC		
		5 days	15 days	25 days	5 days	15 days	25 days
A. ochraceus CCDCA10613	YES	1.28 ± 0.22^{aA}	0.53 ± 1.66^{aB}	0.63 ± 0.23^{aC}	9.95 ± 2.70^{aA}	3.24 ± 1.75^{aC}	9.40 ± 2.03^{aB}
	Coffee	0.22 ± 0.38^{bA}	0.05 ± 0.32^{bB}	0.04 ± 0.02^{bB}	0.12 ± 0.77^{bA}	0.14 ± 0.08^{bA}	0.06 ± 0.02^{bB}
	Wheat	0.03 ± 0.02^{cA}	0.05 ± 0.31^{bA}	0.02 ± 0.34^{cA}	ND	0.02 ± 0.23^{cB}	0.04 ± 0.05^{cB}
	Maize	0.01 ± 0.02^{cA}	ND	0.01 ± 1.76^{cA}	0.01 ± 0.93^{cA}	0.03 ± 0.12^{cA}	0.07 ± 1.23^{bB}
A. ochraceus CCDCA10612	YES	7.05 ± 1.35^{aA}	2.37 ± 1.98^{aB}	1.17 ± 0.75^{aC}	1.20 ± 3.61^{aA}	1.33 ± 0.98^{aB}	2.23 ± 0.84^{aC}
	Coffee	0.10 ± 0.99^{bA}	0.06 ± 0.87^{bA}	0.39 ± 2.08^{bB}	0.05 ± 0.04^{bA}	0.06 ± 0.01^{cA}	0.07 ± 0.98^{bA}
	Wheat	0.04 ± 1.90^{cA}	0.01 ± 0.87^{cA}	0.18 ± 0.98^{cB}	0.01 ± 0.91^{bA}	0.12 ± 1.09^{bB}	0.05 ± 0.02^{bA}
	Maize	ND	ND	0.03 ± 0.09^{dA}	0.01 ± 1.01^{bA}	ND	0.04 ± 2.87^{bA}
A. ochraceus CCDCA10611	YES	0.17 ± 0.94^{aA}	2.83 ± 2.11^{aB}	8.44 ± 3.91^{aC}	7.19 ± 0.89^{aA}	4.61 ± 0.02^{aB}	24.01 ± 3.01^{aC}
	Coffee	0.10 ± 1.01^{bA}	0.08 ± 0.01^{bA}	0.10 ± 0.37^{bA}	0.23 ± 0.55^{bA}	0.33 ± 0.34^{bB}	0.05 ± 0.76^{cC}
	Wheat	0.01 ± 0.62^{cA}	0.04 ± 0.02^{cA}	0.01 ± 0.98^{cA}	0.01 ± 2.78^{cA}	0.07 ± 0.07^{cB}	0.14 ± 0.44^{bC}
	Maize	ND	ND	0.02 ± 0.04^{cA}	0.02 ± 0.29^{cA}	0.02 ± 1.02^{dA}	0.04 ± 1.01^{cA}

*Means followed by the same uppercase (in the row) or lowercase letter (in the column) do not differ from each other by the Scott Knott test at 5% significance. SD: standard deviation; ND: not detected.

carbonarius CCDCA10608 isolate standing out, which showed the highest OTA levels: 29.83 µg/g in YES medium at 15°C and 13.06 µg/g in wheat medium at 15°C. The *A. carbonarius* CCDCA10610 isolate presented a concentration of 8.3 µg/g in coffee extract medium at 15°C. Isolate *A. carbonarius* CCDCA10608 produced 5.07 µg/g in coffee extract medium at 25°C. Treatments

in which it was not possible to detect any OTA level was about 12.5%. Of these undetected treatments, 61.1% are in maize media.

The highest concentrations produced by *A. ochraceus* were in YES medium. Maize culture medium in all treatments had low or no OTA concentration. *A. ochraceus* isolates produced much lower amounts in

Table 2. Ochratoxin A concentration produced by three *A. carbonarius* isolates in different culture media, temperature and incubation time.

Strain	Medium	OTA concentration (ug g^{-1})* ± SD					
		15ºC			25ºC		
		5 days	15 days	25 days	5 days	15 days	25 days
A. carbonarius CCDCA10610	YES	ND	3.61 ± 1.90[bB]	1.00 ± 0.09[bC]	2.96 ± 1.33[aA]	1.05 ± 0.67[cB]	0.12 ± 0.09[cC]
	Coffee	0.05 ± 0.02[aA]	8.30 ± 2.59[aB]	3.21 ± 1.32[aC]	0.03 ± 0.01[cA]	2.33 ± 1.83[bB]	2.50 ± 0.78[bB]
	Wheat	0.02 ± 0.01[aA]	0.02 ± 0.01[cA]	3.65 ± 0.99[aB]	2.33 ± 1.21[bA]	5.22 ± 3.17[aB]	7.52 ± 2.88[aC]
	Maize	ND	0.10 ± 0.09[cA]	0.06 ± 0.01[bA]	ND	0.87 ± 0.29[cA]	0.03 ± 0.01[cB]
A. carbonarius CCDCA10609	YES	0.02 ± 0.01[aA]	0.70 ± 0.09[bB]	0.67 ± 0.08[bB]	1.01 ± 0.48[aA]	0.22 ± 0.09[cA]	0.11 ± 0.06[cB]
	Coffee	ND	0.06 ± 0.02[aA]	0.11 ± 0.03[aA]	0.75 ± 0.09[cA]	0.53 ± 0.22[bB]	0.26 ± 0.11[bB]
	Wheat	ND	0.24 ± 0.13[cA]	0.33 ± 0.04[aA]	0.07 ± 0.06[bA]	4.50 ± 1.02[aB]	6.24 ± 2.97[aC]
	Maize	ND	0.01 ± 0.01[cA]	0.05 ± 0.02[bA]	2.66 ± 0.83[cA]	0.09 ± 0.03[cA]	0.07 ± 0.03[cB]
A. carbonarius CCDCA10608	YES	ND	29.83 ± 1.78[aB]	14.45 ± 4.08[aC]	1.95 ± 0.30[bA]	1.27 ± 0.94[bB]	0.52 ± 0.30[bB]
	Coffee	ND	4.05 ± 1.09[bB]	3.15 ± 1.03[cC]	5.07 ± 1.36[aA]	0.53 ± 0.28[cA]	0.15 ± 0.10[cB]
	Wheat	ND	0.39 ± 0.25[cA]	13.06 ± 1.92[bB]	0.86 ± 0.23[cA]	6.12 ± 2.90[aB]	9.87 ± 3.04[aC]
	Maize	ND	0.06 ± 0.02[cA]	0.41 ± 0.39[dA]	0.07 ± 0.01[dA]	0.12 ± 0.03[dA]	0.02 ± 0.01[cA]

*Means followed by the same uppercase (in the row) - or lowercase letter (in the column) do not differ from each other by the Scott Knott test at 5% significance. SD: standard deviation; ND: not detected.

food-based culture media in comparison with *A. carbonarius* isolates with the maximum production of 0.39 µg/g in coffee extract at 25°C by the *A. ochraceus* CCDCA10612 isolate.

Among all the treatments, it was possible to verify that YES medium was the best substrate for OTA production. The most productive treatment was *A. carbonarius* CCDCA10608; YES; 15°C; 15th day. Regarding food-based culture media, *A. carbonarius* CCDCA10608; wheat medium; 15°C; 15th day stands out as well as the *A. carbonarius* CCDCA10610; coffee extract medium; 15°C; 15th day treatment.

DISCUSSION

Ecological factors such as water activity, temperature and pH have affect OTA production by *A. ochraceus* and *A. carbonarius* (Pardo et al., 2004; Kapetanakou et al., 2011). Studies demonstrate that a higher temperature range allows *A. carbonarius* growth, as in similar studies for proposed optimal growth between 15 and 37°C. (Pitt et al., 2000; Kogkaki et al 2016; Ioannidis et al., 2015). In general, growth under temperatures below 15°C is not common and is only possible at high water activity levels (Bellí et al., 2004). Other studies suggest that the optimal temperature for *A. carbonarius* growth is between 25 and 30°C (Leong et al., 2004) or between 25 and 35°C depending on the isolate and the growth susbtrate used (Mitchell et al., 2003).

Studies conducted by Palacios-Cabrera et al. (2005) demonstrated that temperatures between 25 and 30°C favored *A. ochraceus* colony diameter growth, on the other hand, slower growth could be observed at 35°C and total inhibition at 41°C in all the tested culture media. Substrates directly affect fungal development. YES culture medium is generally considered to be a very favorable medium for OTA biosynthesis (Skrinjar and Dimic, 1992; Bragulat et al., 2001). YES is rich in sucrose, an energy-rich disaccharide with rapid metabolism which may explain the high growth rate of the six tested isolates. *Aspergillus* section *Circumdati* species produce higher amounts of toxin when the carbon source in the medium is sucrose and lower amounts when the sugar available is fructose (Mühlencoert et al., 2004; Medina et al., 2008; Gil-Serna et al., 2015).

Each culture medium may exhibit a different composition, therefore, varying growth rates. With *A. ochraceus,* for instance, the highest growth rates found in optimal conditions were different among culture media in different studies: *A. ochraceus* cultivated in maize-based agar was 3-4 mm/day (Marín et al., 1998), in barley-based medium, 4-5 mm/day, and up to 6mm/day in coffee extract agar medium (Pardo et al., 2005), whereas in the present study, the growth rate was 15 mm/day in YES medium (25°C), 10 mm/day in coffee extract medium (25°C), 10 mm/day in wheat medium (25°C) and 5 mm/day in maize medium. In semisynthetic grape culture medium, *A. carbonarius* showed the highest growth at temperatures of 20 to 33°C, between 0.95 and 0.98 aw, and pH levels between 5 and 6.5. The highest toxin concentration for *A. carbonarius,* 10 µg/g, was found at 15°C, 0.99 aw, and pH 5.35. However, the optimal conditions for toxin production are generally different from those optimal for fungal growth (Passamani et al., 2014).

The combination of factors that presented the highest OTA level was *A. carbonarius* CCDCA10610 in YES medium at 15°C and these data corroborate the results of Mitchell et al. (2004), Bellí et al. (2004) and Leong et al. (2004), who studied different *A. carbonarius* isolates in different interactions, such as the temperature effect, water activity and incubation time, on the production of OTA. Colony development does not follow the same production standards as OTA. For example, on the 5[th] day of incubation at 25°C colonies had already covered almost the entire length of the plates, while isolates incubated at 15°C were developing more slowly, even though the OTA production was generally higher at 15°C. According to Leong et al. (2006), OTA production by *A. carbonarius* and *A. niger* was not related to the growth length, colony size was strongly controlled by temperature, and the toxin was reduced when culture was kept at 30°C. Moreover, maximum OTA production may be related to a specific moment after innoculation due to the strong temperature effect on germination. Nutritional factors are closely related to the activation of genes encoding OTA production. Studies performed in 2009 by Abbas and partners assessed the effect of a wide variability of biotic factors based on nutritional differences regarding OTA and OTB production. Different carbon sources including glucose, sucrose, maltose, galactose, xylose and glycerol seemed to supress OTA production. In contrast, lactose appears to induce OTA production, since the addition of lactose and galactose to the restrictive medium (PDC) resulted in marked increases in OTA levels.

Yeast extract is considered an excellent source of vitamins, amino acids, small peptides, nucleotides, minerals and other nutrients (Zang et al., 2003). In this sense, it is clear that the production of OTA by *A. ochraceus* requires a medium nutritionally rich and appropriate. This validates the results obtained in this present study, where the media most responsive to OTA production by *A. ochraceus* were the Yeast Extract Agar (YES) and Coffee Extract Agar. The unroasted coffee has a wider variety of nutrients than wheat and maize flour, and features more than 300 different compounds, being rich in chlorogenic acids, glycosides, lipids, minerals, caffeine and other compounds (Flament, 2002; Clarke and Macrae, 1988).

In some treatments, there was a decrease in OTA detection from the 15[th] day on. This may occur because some ochratoxigenic fungi may use the OTA produced in the culture medium as substrate, after a certain cultivation time, when nutrients tend to become scarce, in an attempt to find an alternative carbon source. On account of this, strains remove and assimilate the phenylalanine moiety from the OTA molecule like other nitrogen sources in the culture media when they become exhausted (Téren et al., 1996; Varga et al., 2000; Lappa et al., 2015). According to Chalfoun et al. (2000), there was inhibition of OTA production as well as a sporulation decrease by *A. ochraceus* in YES culture media added different caffeine concentrations.

Abrunhosa et al. (2002), observed that most of the strains tested in their study were able to degrade ochratoxin A, whereas 51 strains (67% tested strains) growing in culture medium were able to degrade more than 80% ochratoxin A added to YES medium. In addition, isolates from the genus *Aspergillus (Aspergillus niger, Aspergillus clavatus, Aspergillus ochraceus, Aspergillus versicolor* and *Aspergillus wentii)* stood out as they degraded more than 95% ochratoxin A.

Lower OTA levels were found in treatments with maize-based culture media for both species under study, and the values did not exceed the limit proposed for grain-based food (10 mg/kg) (Agência Nacional De Vigilância Sanitária, 2011), although it is not possible to extrapolate the values of found in tests with culture media to food. These results are in agreement with that of authors who did not detect significant OTA levels in maize silage (Richard et al., 2009; El-Shanawany et al., 2005).

Aspergillus are not usually associated with the production of significant OTA quantities in wheat at lower temperatures (Magan and Aldred, 2007). However, *A. ochraceus* isolates CCDCA0151, CCDCA0153 and CCDCA0162 produced OTA at 25°C in wheat medium, the result corroborates the study carried out by Muñoz et al. (2011) which presented high OTA levels (> 50 µg/g agar) at 25°C in wheat-based culture medium.

The polyketide synthase gene (pks) required in the initial OTA production steps has been, in recent years, widely used in the elucidation of the factors that influence and induce the OTA production by some species of Aspergillus (Gallo et al., 2014). According to O'Callaghan et al. (2006), the culture medium is an important factor in the transcription of this gene and regulation of OTA production, furthermore, culture media supplemented with yeast extract exhibited an increase in PKS transcript levels and OTA accumulation of *A. ochraceus*. Results from the present study with ochratoxigenic strains from *Aspergillus* genus demonstrated a significant OTA production in YES culture medium, but they also produced smaller amounts in coffee extract and wheat-based culture media under the temperature and incubation time conditions tested in this study. *A. carbonarius* CCDCA 10608 isolate in YES medium at 15°C showed the highest OTA levels. Growth rates for all isolates were higher in YES medium at 25°C and the slowest growth took place in maize-based medium at 15°C. Therefore, it is possible to determine, among analyzed variables, the best conditions to avoid or at reduce to diminish OTA production by *A. carbonarius* and *A.ochraceus* in coffee beans, maize and wheat.

Conflict of Interests

The authors have not declared any conflict of interests.

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African Journal of Microbiology Research

Full Length Research Paper

Recovery and prevalence of antibiotic-resistant *Salmonella* from fresh goat meat in Arusha, Tanzania

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Meat products are clearly associated with foodborne pathogens including, antibiotic-resistant strains. Population growth and growing consumer demand facilitate the transmission of foodborne pathogens, particularly in developing countries. To determine the prevalence of antibiotic-resistant *Salmonella* in goat meat, a study was done in Tanzania (June to July, 2015). Overall 120 goat meat samples were collected from five large and five small slaughter facilities (n = 60, respectively). Pre-enrichment for *Salmonella* isolation was done in Tryptic Soy Broth followed by selective enrichment in Modified Semisolid Rappaport-Vassiliadis agar. Isolation of *Salmonella* was done in xylose-Lysine-Deoxycholate agar followed by biochemical confirmation in triple sugar iron agar. The average prevalence of *Salmonella* was 60 and 63% in large and small facilities, respectively. Breakpoint assays indicated an overall low prevalence of resistance (2 to 4%; n = 219 isolates) to ampicillin, amoxicillin, streptomycin, sulphamethoxazole and trimethoprim with complete susceptibility to ciprofloxacin, ceftazidime and cefotaxime. No significant difference ($p > 0.05$) in the prevalence of resistance between large and small facilities was observed. High probability of *Salmonella* contamination of goat meat from Arusha area of Tanzania can pose risks to consumers. Antibiotic resistance appears minimal in this population. Improved hygienic slaughter and meat-handling practices are encouraged to reduce the burden of *Salmonella*-positive meat products.

Key words: Antibiotic resistance, goat meat, *Salmonella*, Arusha, Tanzania.

INTRODUCTION

Meat, including goat meat, is an important source of protein but also can serve as a potential source of foodborne pathogens (Economou and Gousia, 2015).

Meat products are typically contaminated during the slaughter process (Nouichi and Taha, 2009) with pathogens such as *Salmonella* sp., *Campylobacter* sp.,

Listeria monocytogenes and pathogenic strains of *Escherichia coli* (Duffy et al., 2009; Gousia et al., 2011).

Salmonellosis is a common foodborne illness and is caused by a very diverse group of *Salmonella enterica* strains including *S. enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis (de Freitas Neto et al., 2010). Salmonellosis is responsible for approximately 155,000 worldwide deaths annually (Majowicz et al., 2010). Sub-Saharan regions in Africa bear the greatest toll of global foodborne disease burden where 70% of this burden is attributed to non-typhoidal *S. enterica* (Havelaar et al., 2015). People may acquire *Salmonella* infection either by consuming contaminated food products and water, or by direct contact with infected animals (Pui et al., 2011). Antibiotic-resistant *S. enterica* serovar Typhimurium strain type ST313 is a leading cause of bacteremia among African adults and children. In sub-Saharan Africa ST313 infection is associated with a case fatality rate of 20 to 25% (Feasey et al., 2012).

Use of antibiotics in food-animal agriculture is thought to contribute to the emergence and amplification of antibiotic-resistant strains of *Salmonella* (Economou and Gousia, 2015). Although, several published reports illustrate how antibiotic-resistant (ABR) bacteria isolated from food animals overlaps with those causing human infection including foodborne illness (Li et al., 2013; Marshall and Levy, 2011), non-overlap trends of antibiotic resistance infections between humans and animals are also reported (Mather et al., 2012). In developing countries, food and animal products pose a significant risk of transmission for both pathogens and antibiotic-resistant bacteria (Grace, 2015) in part because of unhygienic practices during slaughter and product processing (Mensah et al., 2012). Population growth and rising demand of goat meat in Tanzania for local and export markets has increased total goat meat production from 323,000 tons in 2000/2001 to 449,673 tons in 2009/2010 and consumption projections are still increasing (UNIDO, 2012). This trend may proportionately increase the risk of zoonotic infections associated with food animals in Tanzania. In addition, limited veterinary services and poor husbandry practices might contribute to risks of zoonotic disease transmission (Mellau et al., 2010).

In Tanzania, large slaughter facilities operate mainly in urban centers where meat condition and hygiene practices are inspected by municipal inspectors and health officers, respectively. These operations receive slaughter stock from local traders who purchase the animals either directly from local livestock keepers or obtain the animals from primary and secondary livestock markets. Small slaughter facilities are located in rural areas where inspection of meat and hygiene are uncommon. Small slaughter operators obtain slaughter stock from individual livestock keepers and primary livestock markets (NDO, 2008; UNIDO, 2012). In the Arusha area of Tanzania, roasted goat meat is a popular food-animal product, made famous as *Nyama Choma*. To study the potential contribution of goat meat in the transmission of ABR bacteria to workers and consumers, we estimated the prevalence of ABR *Salmonella* in fresh goat meat obtained from selected large and small slaughter operations in Arusha district.

MATERIALS AND METHODS

Sample collection

Ten (10) goat slaughter facilities were sampled between June and July, 2015. Depending on the number of animals slaughtered per day, the facilities were grouped into five large (A, B, C, D and E; 10 to 150 goat capacity per day) and five small (F, G, H, I and; 1 to 3 goat capacity per day) operations. Meat samples (n = 120, 250 g each) were purchased from five large (n = 60 samples; 12 per facility) and five small slaughter facilities (n = 60 samples; 12 per facility) and were placed in separate sterile polyethylene bags. Two to three samples were collected weekly from each facility over a period of 4 to 6 visits and were transported to laboratory in an ice-cold box within 2 h of collection. In the laboratory subsamples were excised (25 g each) and were washed thoroughly with 25 ml of double-distilled water in sterile plastic bags by soaking and vigorously shaking. An aliquot (1 ml) was transferred into a 2 ml micro-centrifuge tube containing glycerol (15% vol/vol, final concentration) and preserved at -20°C for future use if required. The remaining portion of meat wash was collected in a 15 ml falcon tube for non-selective pre-enrichment of *Salmonella* in the same day.

Isolation and identification of *Salmonella*

An aliquot (1 ml) of meat rinsate was homogenized with 9 ml of Tryptic Soy Broth (TSB, Becton, Dickson and Company, Sparks, MD) and incubated overnight at 37°C to pre-enrich the culture for *Salmonella* (El-Aziz, 2013; Abakpa et al., 2015). After incubation, 30 µl of pre-enriched inoculum was dropped onto Modified Semisolid Rappaport-Vassiliadis (MSRV, Becton, Dickson and Company, Sparks, MD) agar plates containing 2% novobiocin (Becton, Dickson and Company, Sparks, MD). The plates were left to dry for 1 h and were then incubated at 42°C for 18 to 24 h. After incubation, the presumptive motile *Salmonella* were identified by the formation of whitish halos around the drops. Sterile loops were used to pick motile *Salmonella* from the periphery of halos and streak onto Xylose-Lysine-Deoxycholate Agar (XLD, HiMedia Laboratories Pvt, Ltd. Mumbai, India) and Xylose-Lysine-Tergitol 4 (XLT4) agar containing XLT4 agar supplement (Becton, Dickson and Company, Sparks, MD). The XLD and XLT4 plates were incubated at 35°C for 48 h. After incubation, the plates were observed for typical pink to reddish colonies with black centers on XLD plates that were considered presumptive *Salmonella* sp. and yellow to red colonies with black centers on XLT4 plates were identified as considered *S. enterica* serovar Typhimurium. Three isolates per sample were picked with sterile toothpicks and sub-cultured into 96-well plates containing 150 µl of LB broth (LBDifo™, Becton, Dickson and Company, Sparks MD). The plates were incubated at 37°C for 18 to 24 h. After incubation, 40 µl of glycerol (15% vol/vol final concentration) was added. All plates were stored at -20°C (Akbar and Anal, 2014; Lyimo et al., 2016).

Confirmation of *Salmonella* identity by biochemical test

Presumptive *Salmonella* were confirmed by triple sugar iron (TSI) agar (TSI, Becton, Dickson and Company, Sparks, MD) using

previously-described procedures (Addis et al., 2011). Pre-enriched bacterial culture (for each sample in TSB) was inoculated onto TSI agar slants using a sterile inoculating loop and incubated overnight at 37°C. After incubation, colonies that showed yellow and red color changes in the butt and slope, respectively, with blackening of the slope due to production of hydrogen sulfide were confirmed as typical *Salmonella*.

Antibiotic resistance testing in *Salmonella*

Prior to antibiotic testing, the frozen 96-well culture plates were thawed at room temperature for approximately 20 min and a duplicate backup plate was prepared using a sterile 96-pin replicator. Antibiotic breakpoint assays were performed using procedures previously described (Tadesse et al., 2012) and antibiotic concentrations were prepared using recommendations guided by Clinical and Laboratory Standard Institute (CLSI, 2007). The following medically important antibiotics were included in the assays; ampicillin, 32 µg/ml (Amp, VWR International LLC, Sanborn, NY); amoxicillin, 32 µg/ml (Amx, MP Biomedicals LLC); chloramphenicol, 32 µg/ml (Chl, Medaitech Inc); ciprofloxacin, 4 µg/ml (Cip, Enzo Life Sciences Inc); ceftazidime, 8 µg/ml (Cfz, SIGMA-ALDRICH, St. Louis, MO); cefotaxime, 4 µg/ml (Cfx, Chem-Impex International LLC); gentamicin, 16 µg/ml (Gen, Mediatech Inc.); streptomycin, 16 µg/ml (Str, Amresco Inc); sulfamethoxaxole, 512 µg/ml (Sul, MP Biomedicals, LLC); tetracycline, 16 µg/ml (Tet, MP Biomedicals LLC); and trimethoprim, 8 µg/ml (Tri, MP Biomedicals, LLC).

About 1-2 µl of bacterial inoculum (~10^4 CFU per spot) was picked using a sterile 96-pin replicator and transferred onto MacConkey agar (MAC, Becton, Dickson and Company, Sparks, MD) plates containing each antibiotic. The plates were left to dry at room temperature for 15 min and were then incubated overnight at 37°C. After incubation the presence of antibiotic-resistant *Salmonella* was evident when an isolate grew on agar plate containing an antibiotic. *E. coli* K12 was used as negative control (susceptible to all tested antibiotics) and *E. coli* NM-1 (resistant to ampicillin, ciprofloxacin, chloramphenicol, streptomycin, sulfamethoxazole, tetracycline and trimethoprim) and *E. coli* NM-2 (resistant to ampicillin, amoxicillin/clavulanic acid, ceftazidime, ciprofloxacin, kanamycin, streptomycin, sulfamethoxazole, tetracycline and trimethoprim) were used as positive control organisms (Lyimo et al., 2016). NM-1 and NM-2 were originally recovered from water sources in Northern Tanzania and their resistance phenotypes were characterized at Nelson Mandela African Institution of Science and Technology (Arusha, Tanzania) and at Washington State University (Pullman, WA, USA) using the antibiotic breakpoint assays previously described (Rugumisa et al., 2016).

Statistical analysis

All data were summarized by using descriptive statistics and tables and the difference in prevalence of *Salmonella* recovered from goat meat samples was assessed between large and small slaughter operations by using a two-sample Student's t-test. A comparison of prevalence of antibiotic-resistant *Salmonella* within and between large and small facilities across tested antibiotics was complete by using a two-factor analysis of variance (ANOVA) F-statistic and a TukeyHSD *post hoc* test (R software version 3.2.2). All results at *p* < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Salmonella was recovered from >50% of all meat samples with approximately 4% having traits consistent with *S. enterica* serovar Typhimurium. A low prevalence of *Salmonella* Typhimurium was also reported by others in other food animal products such as 11% in beef (Shilangale et al., 2015), 10.8% in cattle and sheep meat and 4.35% in poultry meat (Mezali and Hamdi, 2012). There was no significant difference (*p* = 0.65) in the prevalence between large and small facilities (Table 1). A high prevalence of *Salmonella* contamination in goat meat is consistent with deficits in the procedures used to process goats for meat. Qualitatively, it was observed that goats were held for a longer time in the secondary markets before being transported to slaughter facilities, increasing the chance of animal-animal contamination attributed to excretion while crowding of animals at waiting pans before slaughter might have contributed to more transmission of bacteria. In some areas, slaughter facilities were located closer to live goat auctions, presumably increasing the risk of further transmission. Furthermore, inadequate sanitation and cross-contamination of carcasses through the use of the unclean knives and other utensils for different animal carcasses and possibly the carriage of *Salmonella* sp., by personnel working at these facilities might be another source of carcass contamination.

These observations are consistent to findings reported in Australia by Duffy et al. (2009) who found that the prevalence of *Salmonella* contamination on goat carcasses varied from 3 to 45% in the morning and 20 to 40% in the evening hours, as a result of that which was attributed to poor handling practices. In Ethiopia, a high carriage (80%) of *Salmonella* sp*.,* was reported in various organs of apparently healthy slaughtered goats (Woldemariam et al., 2005) suggesting that carrier animals were potential sources of *Salmonella* contamination in slaughter facilities. In low-income countries like Tanzania, it is common to encounter inadequate food hygiene standards in meat production systems (Roesel and Grace, 2014). For the current study, the prevalence of non-Typhimurium *Salmonella* contaminated goat meat (>50% of carcasses) was higher than other published reports from Pakistan (8%), Ethiopia (3.8%) and Burkina Faso (7%) (Eze and Ivuoma, 2012; Kagambèga et al., 2011; Tadesse and Gebremedhin, 2015). This discrepancy may be attributed to inconsistent slaughter practices but might also be attributed to differences in pre-enrichment and isolation procedures employed across these studies. The low prevalence of serovar Typhimurium isolates (4%) is also consistent with the majority of contamination arising from environmental exposure during slaughter (Maharjan et al., 2006), which is an outcome that can clearly be addressed with greater attention to good hygiene practices. The majority of recovered *Salmonella* isolates (>96%) were susceptible to all of the tested antibiotics (Table 2) with no clear differences (*p* > 0.05) between large and small slaughter facilities (Table 1). Between large facilities, there was no

Table 1. Prevalence (%) of *Salmonella* positive goat meat samples and the % of antibiotic-resistant (ABR) *Salmonella* from selected large and small slaughter facilities in Arusha district, Tanzania.

Slaughter facility	Positive samples (%)	Number of *Salmonella* isolated	ABR (%)
Large facility (n=5)			
A	50	18	11.1
B	58.3	21	28.6
C	50	18	-
D	75	27	29.6
E	66.7	21	4.8
Overall mean	60 ± 4.9^a $(50.5\text{-}69.5)^b$	21.2 ± 1.6^a	18.5 ± 6.2^a
Small facility (n=5)			
F	75	27	7.4
G	75	27	14.8
H	58.3	21	9.5
I	58.3	21	4.8
J	50	18	38.9
Overall mean	63.3 ± 5.0^a $(53.5\text{-}73.1)^b$	22.8 ± 1.8^a	15.1 ± 6.2^a
p-value = 0.65			

[a] Values are means± standard errors; [b] 95% Confidence interval.

Table 2. Average prevalence (%) of antibiotic-resistant *Salmonella* in goat meat from five large and five small slaughterhouses in Arusha, Tanzania; a comparison by two way ANOVA F- statistic test and Tukey HSD *post hoc* test.

Antibiotic	Large facility (n=5)	Small facility (n=5)	Overall mean
Ampicillin	2.2±1.3 (-0.5-4.8)	3.2±1.4 (0.5-5.9)	2.9 ± 0.5^A
Amoxicillin	0.95±0.95 (-0.9-2.8)	0.95±0.95 (-0.9-2.8)	0.82 ± 0.03^A
Streptomycin	4.2±2.1(0.1-8.3)	3.2±2.2 (-1.1-7.5)	3.7 ± 1.1^A
Sulphamethoxazole	1.95±1.3 (-0.5-4.4)	3.6±2.0 (-0.4-7.6)	2.9 ± 0.5^A
Trimethoprim	1.2±1.2 (-1.2- 3.6)	1.1±1.1 (-1.1-3.3)	1.2 ± 0.4^A
Overall mean	2.4 ± 0.7^A	2.2 ± 0.6^A	
F-statistic			
Antibiotic	0.02^{ns}		
Slaughter size	0.38^{ns}		
Slaughter size*antibiotics	0.04^{ns}		

Values are means ± standard errors followed by different superscript letter (s) in the same column or raw which show significantly different groups by Tukey's honestly significant difference *post hoc* test at $P < 0.05$. $^{ns =}$ non-significant ($p > 0.05$). All tested isolates were 100% susceptible to ciprofloxacin, ceftazidime and cefotaxime. Chloramphenicol and tetracycline were not included due to insufficient data.

statistical difference ($p > 0.05$) in prevalence of antibiotic-resistant isolates (Table 3) while for small facilities there was a significant difference ($p < 0.05$) in prevalence that corresponded to a difference ($p = 0.044$) in the rank order of prevalence between sites J and I (Table 4). This difference may be associated with variation in the source of animals entering these facilities or a random difference.

The *Salmonella* isolates recovered for this study were mostly susceptible to antibiotics that are commonly used in Tanzanian livestock production [ampicillin, streptomycin and sulfamethoxazole (Katakweba et al., 2012)] suggesting limited selective pressures from the use of these antibiotics. The limited occurrence of antibiotic-resistant strains in *Salmonella* reported in this study is consistent for what has been reported for slaughtered goats in central Ethiopia where resistance to ampicillin, streptomycin and sulphamethoxazole varied

Table 3. Average prevalence (%) of antibiotic-resistant *Salmonella* in goat meat samples from different sites within large slaughterhouses in Arusha district, Tanzania; a comparison by two-way ANOVA F-statistic and Tukey HSD *post hoc* test

Large facility (n=5)	Antibiotic[a]					Overall mean
	Amp	Amx	Str	Sul	Tri	
A	0.53±0.53	0.53±0.53	-	-	-	$0.21±1.5^A$
B	-[b]	-	1.6±1.6	-	-	$0.32±0.32^A$
C	-	-	-	-	-	-
D	0.61±0.61	-	0.61±0.61	0.61±0.61	0.61±0.61	$0.49±0.24^A$
E	-	-	0.46±0.46	0.46±0.46	-	$0.19±0.13^A$
Overall mean	$0.26 ±0.18^A$	$0.11 ± 0.11^A$	$0.49 ± 0.31^A$	$0.23 ±0.17^A$	$0.14 ±0.14^A$	
F-statistic						
Antibiotic	0.59^{ns}					
Site	0.86^{ns}					
Antibiotic*Site	0.70^{ns}					

Values are means ± standard errors followed by different superscript letter (s) in the same column or raw which show significantly different groups by Tukey honestly significant difference post *hoc* test at $p < 0.05$. [a] Amp, ampicillin; Amx, amoxicillin; Str, streptomycin; Sul, sulphamethoxazole and Tri, trimethoprim, All tested isolates were 100% susceptible to ciprofloxacin, ceftazidime and cefotaxime. -[b] = not detected; [ns] = non-significant ($p > 0.05$).

Table 4. Average prevalence (%) of antibiotic-resistant *Salmonella* in goat meat samples from different sites within small slaughterhouses in Arusha district, Tanzania; a comparison by two-way ANOVA F-statistic and Tukey HSD *post hoc* test.

Small facility (n=5)	Antibiotic[a]					Overall mean
	Amp	Amx	Str	Sul	Tri	
F	0.41±0.41	-	-	0.41±0.41	-	$0.21±0.12^{ABC}$
G	0.25±0.25	-	-	0.13±0.13	-	$0.32±0.19^{AC}$
H	-[b]	0.18±0.18	-	-	-	$0.11±0.11^{AB}$
I	-	-	0.16±0.16	-	-	$0.09±0.09^B$
J	0.21±0.21	-	0.42±0.42	0.42±0.42	0.21±0.21	$0.93±0.42^A$
Overall mean	$0.41 ±0.24^A$	$0.12 0.12^A$	$0.41 ± 0.31^A$	$0.47 ±0.31^A$	$0.14 ±0.14^A$	
F-statistic						
Antibiotic	0.40^{ns}					
Site	2.5^{*c}					
Antibiotic*Site	0.49^{ns}					

Values are means ± standard errors followed by different letter(s) in the same column or row designate significantly different groups by Tukey's honestly significant difference post hoc test at $p < 0.05$. [a] Amp, ampicillin; Amx, amoxicillin; Str, streptomycin; Sul, sulphamethoxazole and Tri, trimethoprim. All tested isolates were 100% susceptible to ciprofloxacin, ceftazidime and cefotaxime. -[b] = not detected; [c] significant at $p < 0.05$; [ns] = non-significant ($p > 0.05$).

between 4.6 and 18.2% (Molla et al., 2006). In contrast, a high prevalence of antibiotic-resistant *Salmonella* to ampicillin (54.5%, amoxicillin (45.5%), streptomycin (81.8%), sulphonamide (42%) and Trimethoprim (75%) was reported for goat meat in eastern Ethiopia (Ferede et al., 2015). Presumable, these differences reflect differences in exposure to antibiotics, bacterial diversity and selection pressure in the natural environment (Sharma and Bist, 2010). Importantly, while a very low prevalence of antibiotic-resistant *Salmonella* in goat meat from Tanzania is observed, growing consumer demand in Tanzania (UNIDO, 2012) will result in increased production efforts and potentially an increasing reliance

of antibiotics for food animal production and a potential commensurate increase in the prevalence of antibiotic-resistant *Salmonella*. Examples include reports of increased prevalence of *Salmonella* from poultry and beef that are resistant to fluoroquinolones and third-generation cephalosporins (Ahmed et al., 2014; Cabrera-Diaz et al., 2013; M'ikanatha et al., 2010). The increased use of antibiotics during animal breeding can introduce a selective pressure that leads to the development of resistance or even multi-resistance characteristics in some bacterial populations (Chen and Jiang, 2014).

Occurrence of *Salmonella* isolates that were resistant to ≥1 antibiotic was uncommon (10.5% of 219). Of these,

the most prevalent multidrug resistance at larger facilities included resistance to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole and tetracycline (7.8%) and resistance to ampicillin, sulfamethoxazole and tetracycline (5.7%) in small facilities. This is dramatically different from the prevalence of multidrug resistant isolates (≥1 antibiotic) recently reported from water sources in northern Tanzania (88.5%) (Lyimo et al., 2016). If the lack of overlap in antibiotic resistance is an appropriate metric, then it is likely that goats are not a significant contributor to *Salmonella* contamination in these waters. In addition, the prevalence of multi-drug resistant *Salmonella* observed in this study is lower compared to prevalence reported from other food animals in Sudan (33.3 to 66.6%), Ethiopia (93.2%), United States (62%) and Korea (87.2%) (Fadlalla et al., 2012; Ferede et al., 2015; Khaitsa et al., 2007; Kim et al., 2012). This difference may suggest low antibiotics consumption in goat populations raised from Arusha area. Further studies are required to directly assess the impact of this practice. The present study indicates a high probability (>50%) that goat meat carcasses from the Arusha area are contaminated with *Salmonella.* Fortunately, antibiotic resistance strains were detected infrequently. Nevertheless, producers, processors and consumers should be alerted to better carcass and product handling practices to minimize the risk of *Salmonella* transmission from these products.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Abbreviations

ABR, Antibiotic resistance; **CLSI,** Clinical and Laboratory Standard Institute; **NDO,** Netherlands Development Organization; **NMAIST,** Nelson Mandela African Institution of Science and Technology; **UNIDO,** United Nation Industrial Development Organization.

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