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

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

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African Journal of Microbiology Research

Review

The basis of nonhost resistance for future genetic engineering to find out durable resistance in agricultural crops

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Plant disease resistance is one of the most desirable traits for agricultural production, especially in the present time of fear over food production and crop security. Disease plays an important role in crop production and quality of products. The one key factor of food security and production is plant disease resistance. Several resistance gene(s) are reported from the same host range to overcome against disease resistance. These resistance (R) genes are not durable in many cases because of rapid changes in the pathogen population to overcome the resistance that they confer. For diagnosing such type of situation, continuous search of durable resistance sources from across the genera/species are desirable. Second type of resistance that is nonhost resistance has been described as inaccessibility. Nonhost resistance is regarded as a robust protection against pathogenic microorganisms because of its durability. The mechanisms of nonhost resistance could also be exploited to improve the resistance in a range of crop plants. Recently several components of nonhost resistance have been identified but such resistance is one of the least understood phenomenons in the area of plant microbe interaction. Molecular mechanism of nonhost resistance is not fully understood. Though, nonhost resistance will help biologist to engineer the plants for more durable resistance against important plant diseases. Therefore, non-host resistance seems to be one avenue under consideration for significant improvement of agriculture production in future.

Key words*:* resistance gene, hypersensitive response, leucine rich repeat.

INTRODUCTION

Plants are sessile organisms, incapable of feeling the possibly harmful microorganisms. Defense of plants against pathogen infections are broadly based on diverse strategies. Plants possess a range of constitutive or inducible resistance mechanisms to defend themselves against the particular pathogenic attack. For this, plants may have evolved mechanisms to perceive pathogen attacks and to translate that perception into an adaptive response. In contrast to animals which possess specialized cells for defense, each individual plant cell possess

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Abbreviations: HR, Hypersensitive response**; Avr,** Avirulence; **NHR,** nonhost resistance; **TTSS,** Type III secretion system.

a preformed inducible defense capability for their protection. Host resistance is shown by a particular cultivar within a plant species that as a whole is normally susceptible to the pathogen. On the other hand, nonhost resistance is expressed by all members of a plant species to a particular pathogen. However, even susceptible plants are capable of reacting in a way that may slow down growth of the pathogen. The presence of such basal resistance was evidenced in *Arabidopsis* by the isolation of enhanced disease susceptibility mutants (Heath, 2000). Extensive studies on gene for gene resistance have made it possible to clone > 40 Resistance (R) genes from plants (Martin et al., 2003). Such single dominant R genes can be transferred within closely related species to protect the more agronomically useful crop plants. Resistance conferred by single dominant R gene is specific to a particular pathogen race that can express the corresponding avirulance gene(s). Pathogen avirulance genes can be easily mutated or eliminated, and hence protection conferred by R genes is not durable.

Drawing a discrete line between host and nonhost status is not always straight forward, since some plant and pathogen that is species combinations suggest marginal host or near-nonhost status, when only few accessions of a plant species are at most moderately susceptible to a heterologous pathogen (Niks, 1987). It is generally concluded that the majority of plants are immune to the majority of microbes with pathogenic potential against the pathogen those can infect two plant species in a habitat (Yun et al., 2003). Nonhost resistance (NHR) is displayed at the species level that is all cultivars of a plant are resistant to infection by all genotypes within a pathogen species. Though nonhost resistance still remains poorly understood, but some important progress has been made in the recent year. Studies on the genetics of plant NHR identified critical components mediating cross-species resistance (Heath, 2002).

Due to the durable immunity, the molecular mechanism of nonhost resistance in plants outside the host range of a particular pathogen has attracted much attention earlier, which exerts broad-spectrum activity against the different disease causing species. NHR, however, has proved difficult to characterize as a result of the absence of a tractable genetic system. It is thought to be genetically complex, involving the deployment of both constitutive and inducible defense responses, in combination with a host physiology that may be routinely incompatible with pathogenesis. The present review is focused on recent advances of nonhost resistance, its mechanism and components involved in nonhost resistance towards plant systems.

BASIS OF HOST AND NONHOST RESISTANCE

Broad spectrum of host and non-host resistance

Resistance to different diseases of plants has historically been classified into two major categories, (i) host and (ii) nonhost resistance. Host resistance to plant pathogen has been more thoroughly investigated, unlike non-host resistance, because it is genetically accessible (Gilbert and Webb, 2007). This general case is termed as host resistance, specific resistance, genotypic resistance, or cultivar resistance. Such resistance occurs when genetic polymorphism for susceptibility is observed in the same plant taxon, i.e., some genotypes show heritable resistance to a particular pathogen whereas other genotypes in the same gene pool are susceptible. In resistant individuals, the pathogen may or may not multiply to some extent, but spread of the pathogen through the plant is demonstrably restricted relative to susceptible hosts, and disease symptoms generally are either highly localized or are not evident. Host resistance, however, is usually restricted to a particular pathogen species and is commonly expressed against specific pathogen genotypes. In this case, plant specifically recognizes the invading pathogen and active defense responses are induced that lead to resistance. Elicitation of defense responses is mediated by the perception of pathogen signal molecules encoded by avirulence *(Avr)* genes only when the matching plant resistance *(R)* gene is present, which results in an incompatible interaction between resistant host and avirulent pathogen. If the *R* and/or *Avr* gene is absent or nonfunctional, the interaction between host (susceptible) and pathogen (virulent) is compatible. As opposed to the basal defense responses that often partially inhibit pathogens during colonization of the host plants, *R*-gene-mediated resistance involves a rapid and effective defense (Figure 1). Plant defends itself, as resistant (immune) reaction when it comes in contact with a pathogenic agent to which the plant is not a host (Figure 1). This is known as nonhost resistance and is the most common form of resistance (or defense from attack) in nature and unsuccessful plant/pathogen interactions represent nonhost resistance (Fraser, 1990). In nonhost resistance, all genotypes within a plant species show resistance or fail to be infected by a particular pathogen, specifically signifies the state where genetic polymorphism for susceptibility to a particular pathogen has not been identified in a host taxon. Although underlying mechanisms of nonhost resistance to pathogens are largely unknown and are likely as diverse for pathogen as they are for other classes of plant pathogens (Agrios, 1997). For example, mango tree will not be infected when the pathogens of tomato, of wheat, or of citrus trees come in contact to the above said plants, because the genetic makeup of mango tree is different to that of the host plant (tomato, wheat or citrus); are incompatible to each other and under that case due to that genetic constitution and needs specific reaction site for induction of disease/ specificity. Similarly, the fungus that causes *Fusarium* wilt on pigeon pea (*Fusarium udum*), does not infect pigeon pea, and so on unless it posses specificity for susceptible host. Understanding the ways by which infection fails to nonhost plants may be particularly important for break throughs in the development of plants with durable broad-

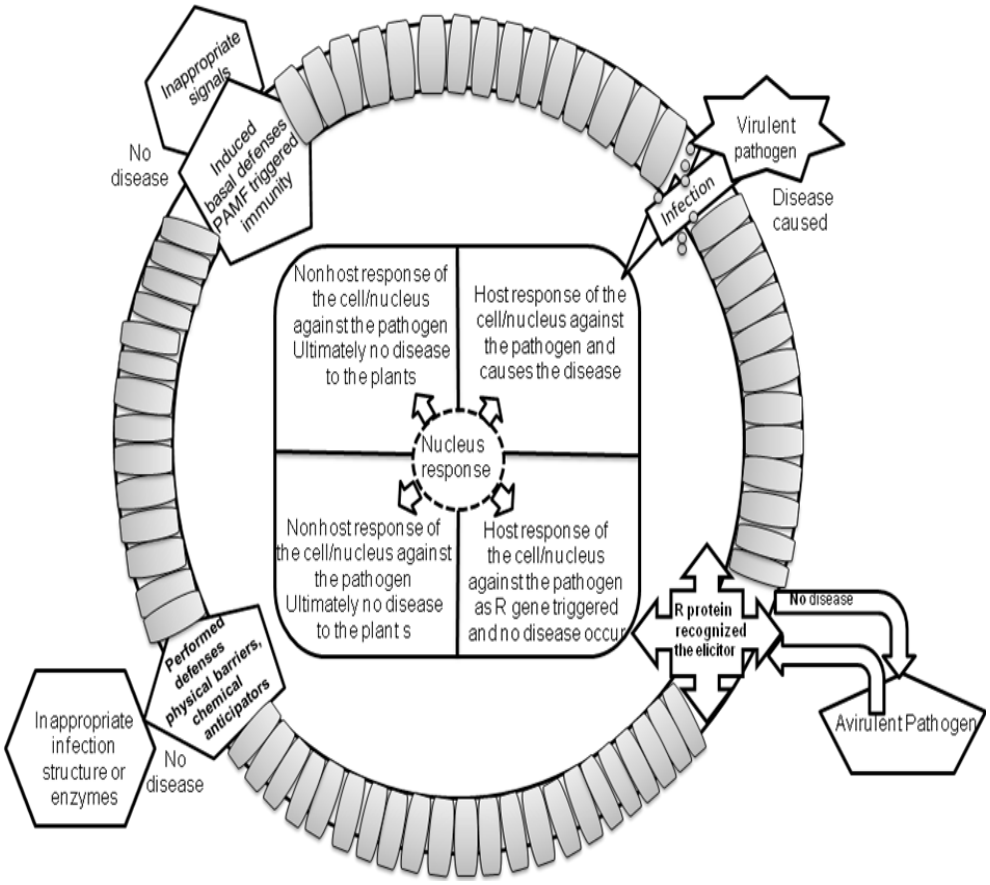

Figure 1. Interaction and disease development phenomenon in plant by host resistance and nonhost resistance

spectrum disease resistance.

CLASSES OF NONHOST RESISTANCE

The type of nonhost resistance depend how plants triggered in a resistance response and this depends on both the plants and pathogen species. Based on the observations recorded earlier, nonhost resistance against bacteria, fungi and oomycetes was classified into two types: type I and type II, however, it is still not clear that non-host resistance against viruses can be classified in the same manner (Mysore and Ryu, 2004) or a new classification have to be evolved for viruses. It is also reported that different plant species may show different resistance response by a single pathogen as both non-host resistances type I and type II. This may be due to the fact that type I and type II are mainly resisted by the passive mechanism when essential host components are missing in the pathogen (Holub and Cooper, 2004). Tobacco and *Arabidopsis* mutant analysis also revealed that some of the signal transduction pathways of type II NHR might converge with host resistance pathways (Yun et al., 2003).

Type I nonhost (non HR mediated) resistance

Type I nonhost resistance is the most common type of non host resistance in plant system till date and this type of resistance does not produce any visible symptoms (necrosis or surface injury) on the infected plants. The pathogen will not be able to get pass the first or the second obstacle of the plant cell, and finally the multiplication and penetration of the pathogen into the plant in type I nonhost resistance will be completely arrested at the site of pathogen invasion. Though in this reaction the plant looks normal from their outer surface (without any visible symptoms), but inside the plant cell, several molecular changes might be happening. In some cases, nonhost resistance is not associated with induction of a hyper sensitive response, such that a nonhost plant species can show type I nonhost resistance against one pathogen species and type II resistance against another pathogen species. For example, *Nicotiana benthamiana* exhibits type I nonhost resistance against *Xanthomonas campestris* pv. *campestris* and type II nonhost resistance against *Pseudomonas syringae* pv. *tomato*. For example, *P. syringae* pv. *phaseolicola* triggers type I nonhost resistance in *Arabidopsis* and type II nonhost resistance

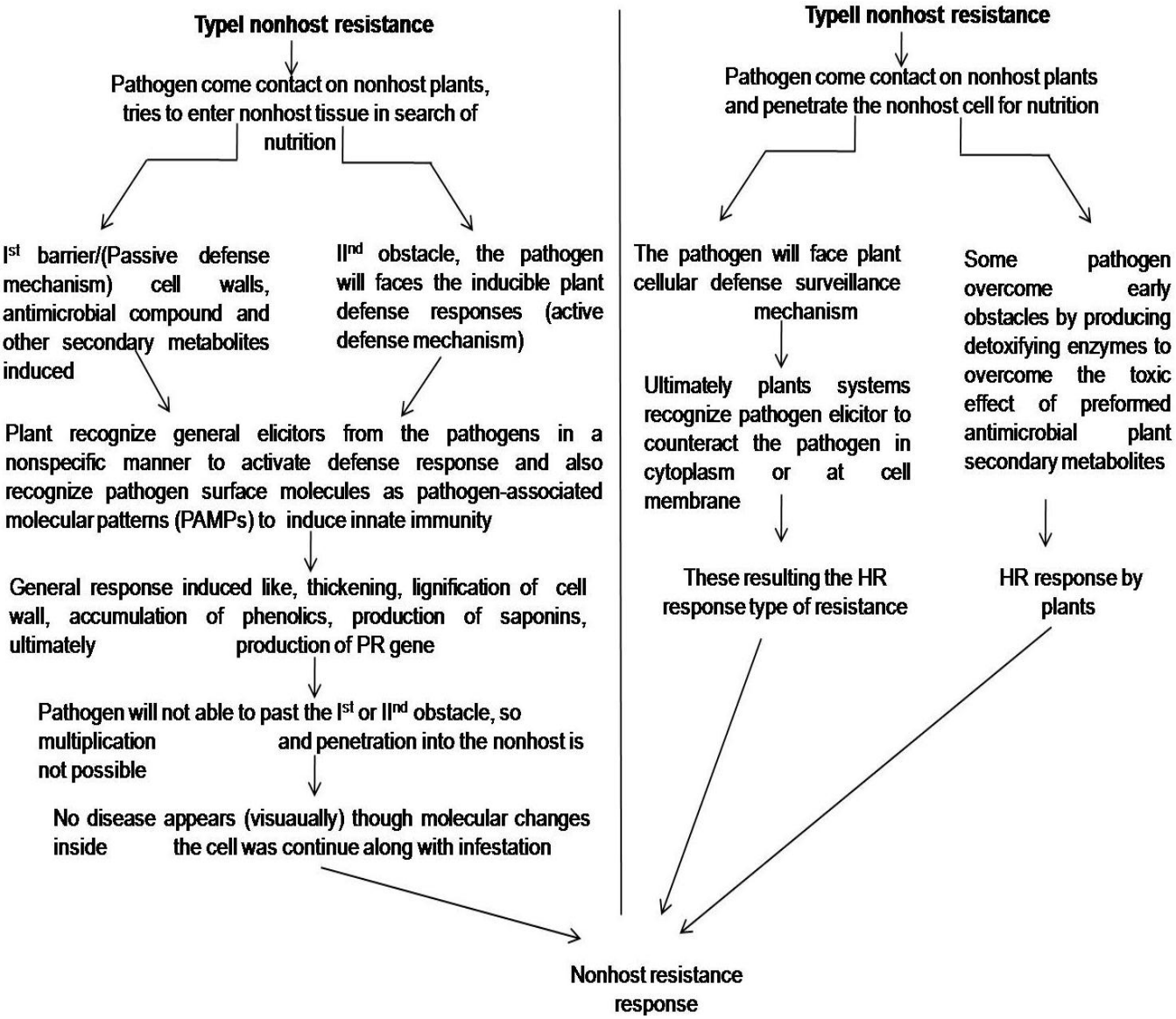

Figure 2. **A**. Resistance mechanism of the nonhost I, **B**. Resistance mechanism of the nonhost II to resist the pathogen.

in tobacco (Dawson and Hilf, 1992). In Figure 2A, the general model for the mechanism of type I (non-HR-mediated) nonhost resistance is given.

Type II nonhost (HR mediated) resistance

Type II nonhost resistance is a more sophisticated plant defense mechanism than type I nonhost resistance and phenotypically similar like as an incompatible gene-for-gene interaction. Unlike to type I nonhost resistance, type II nonhost resistance is always associated with rapid localized necrosis (HR). An elicitor(s) will be recognized by the plant surveillance system and a defense reaction leading to HR will be activated. This will prevent the further spread of the pathogen from the infected cell (Figure 2B). Similarities were reported between nonhost as well as in gene for gene resistance responses but it is still not clear what mechanisms are involved in producing these resistance responses. It has been reported that some pathogens can conquer early obstacles by producing detoxifying enzymes to overcome the toxic effect of preformed antimicrobial plant secondary metabolites (Lu et al., 2004). In the second phase of the plants, when it faces the pathogen, then the plant internal cellular system goes to defense surveillance. After that the plants have evolved to recognize certain pathogen elicitors, either in the plant cytoplasm or at the plant cell membrane, a defense mechanism is triggered to hyper sensitive response. Such type of pathogen elicitors can be recognized by plants to activate defense responses known as avirulence (*Avr*) proteins. Avr proteins when not recognized

by plants can enhance the virulence of pathogens (Osbourn, 1996). Once a pathogen can overcome preformed and general elicitor induced barriers, fungal and oomycete pathogens can directly penetrate a plant cell whereas most plant bacterial pathogens inject virulence and avirulence proteins into the plant cell through a *hrp* gene-encoded type III secretion system (TTSS). For fungal and oomycete pathogens, the extracellular proteins on the hyphae or secreted proteins serve as elicitors whereas the injected avirulence proteins serve as elicitors for bacterial pathogens (Shan et al., 2004).

MECHANISM OF RESISTANCE AND EXAMPLE OF NONHOST RESISTANCE

Mechanism and examples of nonhost resistance

In general, a pathogen lands on a nonhost plant, then it tries to enter the host tissue in search of nutrition for its biological activity. After landing, the pathogen will face plant barriers (known as passive defense mechanisms), the first barrier like cell walls, second antimicrobial compounds and other secondary metabolites (Hutcheson et al., 2001). Third barrier against the pathogen is the inducible plant defense responses (known as active defense mechanisms). After induction of the third barriers in plants, it recognizes general elicitors from pathogen population in a nonspecific manner to activate its defense machinery. In other way, plants can also recognize pathogen surface molecules referred as pathogen-associated molecular patterns (PAMPs), to induce innate immunity. PAMPs are shared among members of a pathogen group and are known to induce innate immunity in both plants and animals systems. Some of the plant defense responses that are induced because of general elicitors and PAMPs include cell wall thickening, cell wall lignification, and accumulation of phenolics, production of saponins, and production of phytoalexins, papilla formation and induction of pathogensis related genes (Thordal-Christensen, 2006). Like the host systems, nonhost interactions with pathogens can also mount/ induced a battery of induced barriers against pathogen infection. Arabidopsis is a nonhost for *P. syringae* pv. *phaseolicola* and when infected with *P. syringae* pv. *phaseolicola*, it activates PR gene expression without any visible symptoms (Dawson and Hilf, 1992).

Specific resistance has been extensively studied in host pathosystems and typically follows Flor's gene-for-gene model (Dixon, 2001), in which resistance is determined by the simultaneous expression of pathogen avirulence (*Avr*) gene and the corresponding plant resistance (R) gene. Most contemporary models of non-host resistance evoke a complex overlay of specific resistance and nonspecific defense responses (Staskawicz et al., 1995). However, the extent to which the gene-for-gene model applies to non-host interactions remains unclear. A classical genetic approach to this problem is hampered by the absence of variation in plant resistance and pathogen virulence, as well as by sexual incompatibility between host and non-host plants. Specific resistance has been extensively studied in host pathosystems and typically follows Flor's gene-for-gene model. Resistance is determined by the simultaneous expression of pathogen avirulence (*Avr*) gene with the corresponding plant resistance gene, leading to the hypersensitive response (HR), a general defense response of plants that includes apoptotic cell death (Nurnberger and Brunner, 2002). The extent to which the gene-for-gene model can be expanded to non-host interactions remains unclear. However, in many pathosystems, non-host resistance can be explained by the occurrence of an arsenal of R genes that recognize multiple or essential *Avr* genes (Dangl and Jones, 1998). Although historically, the above classifications have been quite stringent, plant pathologists have recently begun to appreciate major genetic and molecular intersections between the various types of plant immunity. For example, genes such as NPR1 and EDS1/ PAD4, which encode key components of salicylic acid mediated plant defense signaling, were found to be essential for various types of disease resistance (Kamoun, 2001). Likewise, global transcription profiling revealed similar sets of genes whose expression is altered during basal defense, R gene-triggered immunity and in non-host interactions (Lipka et al., 2005; Caldo et al., 2004). NB-LRR and PRR-triggered immunity contribute to nonhost resistance such that with increasing phylogenetic divergence time between two plant species, the relative effectiveness of PRR-triggered immunity increases whereas the relative contribution of NB-LRR protein-triggered immunity decreases (Caldo et al., 2004; Zimmerli et al., 2004). The other one mechanistic explanation for this could be that effectors from a given pathogen species fail to effectively suppress PRR-triggered immunity in nonhosts because their corresponding host cellular targets have diverged in the nonhost to an extent that impedes effective manipulation by the effector repertoire. Additionally, the continuous co-evolutionary arms race between host-adapted pathogens and their hosts appears to drive a more rapid evolution of NB-LRR loci compared to the rest of plant genomes (Schulze-Lefert and Panstruga, 2011).

IDENTIFICATION OF NONHOST RESISTANCE GENE(S) AND NEW ADVANCEMENT IN THROUGH MOLECULAR TOOLS

Identification and advancement in nonhost resistance through molecular tools

Mutants of Arabidopsis (*A. thaliana*) are used for the identification of several genes that contribute to nonhost resistance against the barley powdery mildew fungus *Blumeria graminis* f. sp. *hordei*. The study then hypothe-

sized that the multilayered nonhost resistance was found in plants, with the plant cell wall being the first and rapid cell death the second line of defense. In wild-type plants, inappropriate pathogens to which *Arabidopsis* is a nonhost are usually stopped at the preinvasive stage of penetration. This penetration resistance is associated with the formation of large cell wall appositions (papillae) enriched in callose, lignin-like material, and hydrogen peroxide (Leister et al., 1998). Upon breaching of this first defense layer, pathogen growth is stopped by a hypersensitive reaction of attacked cells, which is associated with auto fluorescence and a hydrogen peroxide burst and which leads to cell death (Stein et al., 2006). Recently, it has been shown that genes of Arabidopsis identified to play an important role in nonhost resistance against powdery mildews also contribute to resistance against nonhost rust fungi such as *Phakopsora pachyrhizi* (Schweizer, 2007). *Arabidopsis* NahG plants have a defect in resistance to the non-host bacterial pathogen *P. syringae* pv. *phaseolicola* NPS3121 (*Psp*), suggesting that SA-mediated signaling is required for non-host resistance to this pathogen. In light of the phenotypic differences between NahG and SA-signaling mutants (Loehrer et al., 2008), further investigation of the role of SA signaling in nonhost resistance to Psp observed that resistance of Arabidopsis to Psp was affected only in NahG plants and not in other genotypes with defects in SA signaling (Lu et al., 2001). Therefore, nonhost resistance to Psp is independent of SA signaling. In the nonhost interactions of barley with inappropriate rust fungi, a better understanding of the genetic basis of nonhost resistance was achieved recently by accumulating susceptibility alleles in a series of consecutive crosses, which resulted in two barley lines with essentially full susceptibility to nonhost rusts (Wees and Glazebrook, 2003). It was concluded, therefore, that nonhost resistance, at least to rust fungi, might depend on a complex and functionally redundant set of genes in barley. Barley (*Hordeum vulgare* subsp. *vulgare*) has been reported to be a nonhost to the wheat powdery mildew fungus *Blumeria graminis* f. sp. *tritici*, the wheat leaf rust (Rust) fungus *Puccinia triticina*, and isolate CD180 (CD) from the genus *Magnaporthe* that is associated with the host *Pennisetum* species (Atienza et al., 2004).

A maize R gene recognizes a rice pathogen, *Xanthomonas oryzae* pv. *oryzicola*, which causes bacterial streak disease in rice. Although *X. o.* pv. *oryzicola* does not cause disease on maize, though identified *Rxo1* gene, a maize plant gene, conditions a resistance reaction to a diverse collection of pathogenic strains of *X. oryzae.* pv. *oryzicola*. Surprisingly, *Rxo1* also controls resistance to the unrelated pathogen *Burkholderia andropogonis*, which causes bacterial stripe of sorghum and maize. The same gene also controls resistance reactions to both host pathogens and nonhost pathogens of maize. *Rxo1* has a nucleotide-binding site leucine-rich repeat structure, similar to many previously identified R genes (Zellerhoff et al., 2006). Most importantly, *Rxo1* functions after transfer as a transgene to rice, demonstrating the feasibility of nonhost R gene transfer between cereals and providing a valuable tool for controlling bacterial streak disease. The function of *Rxo1* in rice demonstrates that an NBS-LRR type of R gene can be effectively transferred between distantly related cereals. In similar experiments, maize *Rp1* genes, which are also NBS-LRR genes, did not function in wheat and barley (Ramakrishna et al., 2002). Other NBS-LRR R genes also have a restricted taxonomic functionality (Zhao et al., 2005), although some aspects of their function can sometimes be detected after transfer between distantly related species (Tai et al., 1999). If other R genes function in heterologous cereal backgrounds, it will have a significant impact on strategies to improve disease resistance in most crops. Considering that the NBS-LRR genes account for >1% of genes in the rice genome (Frost et al., 2004) and the thousands of different species of grasses, this could provide a deep resource of R genes to control different phytopathogens. In the case of *Rxo1*, the maize gene confers broad resistance to a diverse group of *X. oryzae.* pv. *oryzicola* strains in rice (Monosi et al., 2004), where no R genes for this disease are known in host species. Some of the identified nonhost specific genes have novel functions when compared with known host-specific resistance and defense-related genes, respectively, revealing the existence of separate signaling and effector components (Mysore and Ryu, 2004). Some insights were also gained on the nature of elicitors leading to host species-specificity of *Magnaporthe grisea* (Murakami et al., 2003) and *X. oryzae* (Sweigard et al., 1995) suggesting that in some cases major genes appear to control host specificity and are likely to be recognized by specific plant receptors, yet to be identified.

Molecular mechanisms underlying HR of rice to its bacterial pathogen *Xoc* is mediated by a nonhost maize R gene. *Rxo*1 were investigated using a microarray experiment and a pair of transgenic and non-transgenic rice lines. *Rxo*1 appeared to function in the very early step of the interaction between rice and *Xoc*, and could specifically activate large numbers of genes involved in signalling pathways leading to HR and some basal defensive pathways such as SA and ET pathways. In the former case, *Rxo1* appeared to differ from the typical host R genes in that it could lead to HR without activating NDR1. In the later cases, *Rxo*1 was able to induce a unique group of set of WRKY transcription factor genes and a large set of genes encoding PPR proteins that share the same G-box in their promoter regions with possible functions in post transcriptional regulation. Some key genes that function in the downstream of *Rxo*1 were identified, including OsNPR1 and OsPR1. Results elucidated some interesting aspects on the molecular mechanism of the non-host resistance of rice mediated by Rxo1 and provided useful information to understand the evolution of

plant resistance genes (Zhao et al., 2004; Zhou et al., 2010).

Progress has been made in the model plant Arabidopsis, to a better understanding of the genes and pathways underlying nonhost resistance. The molecular basis of nonhost resistance is of obvious interest due to its durability, and potential transfer of this resistance mechanism(s) by transgenic approach to crop plants that are hosts of a given pathogen species will give a tool to better resistance. Future, research to find additional nonhost resistance gene against the important pathogens requires inter discipline approaches of genomics, structural biology, advanced genomics and bioinformatics tools. Genomics approaches will contribute by identifying candidate nonhost gene(s) and their determination of the positive selection. Structural biology is essential to understand the mode of interaction and inhibition of the pathogen. Advanced proteomics includes not only the sensitive pull down array to identifying and interaction but also activity based protein based assay for the determination of pathogen and non-host interaction. The potential of finding additional non-host gene(s) for the important pathogens is tremendous for better disease resistance. For all given the clear action of mechanism of action of the non-host resistance may be the additional layers of manipulation of several plant species with the molecular battlefield to the non-host resistance.

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Review

Probiotic encapsulation

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Encapsulation technologies are used to keep probiotic cell viable throughout storage, commercialization and use in food products, so that these cells are active during their passage through the gastrointestinal tract. This review describes the most common encapsulation technologies and encapsulation materials used for maintaining the viability of probiotic bacteria under adverse external conditions. Illustrations are presented to facilitate the understanding of the various encapsulation methodologies. Supercritical fluid technologies for encapsulation as well as new wall materials are discussed. It is concluded that several variables affect the viability of encapsulated probiotic cells and therefore optimization tools including response, genetic algorithms, quadratic sequential programming are needed for appropriate material selection.

Key words: Lactic acid bacteria, lyophilization, spray drying, extrusion, cellular viability.

INTRODUCTION

Probiotics, from the Greek word meaning "for life", are defined as living organisms that provide health benefits to the host when ingested in sufficient quantities (Quigley, 2010). Lactic acid bacteria (LAB) are the most commonly used probiotic microorganisms due to their beneficial effects on the gastrointestinal tract. Probiotic bacteria are used in the food industry due to various beneficial properties including reduction of irritable bowel syndrome symptoms after *Bifidobacterium infantis* 35624 intake, immunomodulatory effects, and cholesterol reduction (FAO/WHO, 2006).

The administration of *Lactobacillus rhamnosus* LGG during acute rotavirus diarrhea in children, decreased the diarrhea duration vs. placebo (Jankovic et al, 2010); however, the use of other LAB (e.g., *Lactobacillus johnsonii*) was associated with gastritis by *Helicobacter pylori* (Pantoflickova et al., 2003).

Antibiotics are used in cattle to treat bacterial infections and to promote growth when used in low concentrations. However, the widespread use of antibiotics caused resistance to pathogenic bacteria. Probiotic organisms are good alternatives to the widespread use of antibiotics (Edens, 2003). The gastrointestinal tract of calves is sterile at birth, and organisms are introduced from vaginal and fecal microbiota, and from the environment (Rosmini et al., 2004).

In all cases, probiotic bacteria should remain alive from the time they are consumed until their settlement in the intestine. This is difficult since the bacteria must bypass extreme acidic pH in the gastrointestinal tract. Encapsulation of probiotic bacteria is an alternative that provides protection for living cells exposed to an adverse environment (Burgain et al., 2011). It also helps food materials to resist processing and packaging conditions, improving taste, aroma, stability, nutritional value and product appearance (Parra-Huertas, 2010).

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Probiotic encapsulation of LAB has been proposed as an efficient technology to improve viability and preserve metabolic activity in the gastrointestinal tract (Picot and Lacroix, 2004), and to ensure viability during long-term storage (Zuidam and Nedovic, 2010). Viability is defined as the number of trapped (encapsulated) probiotic cells (cfu g^{-1}) that remain viable in their site of action to produce a beneficial health effect to the host (Krasaekoopt et al., 2003; De Vos et al., 2010). Encap-sulation has been successfully used to improve cell viability during storage of several LAB including *Lactobacillus paracasei* NFBC 338 by spray-drying (Desmond et al., 2002), *Lactobacillus casei* NCDC-298 by emulsification (Mandal, et al., 2006) and *L. casei* by extrusion (Sandoval-Castilla et al., 2010), among others.

This review on probiotic encapsulation discusses materials and techniques used for encapsulation, and factors that affect the viability and controlled release of cells. New potential research avenues are also briefly discussed.

ENCAPSULATION

Encapsulation is defined as a process that entrap a substance into another substance, producing particles in the nanometer (nanoencapsulation), micrometer (micro-encapsulation) or millimeter scale (Lakkis, 2007; Burgain et al., 2011).

The encapsulated substance is usually called core material, active agent, filler agent, internal phase, or payload phase. A substance used to encapsulate is called coating membrane, shell, carrier or wall material, external phase or matrix. For specific encapsulation processes such as freeze-drying, the substances used to encap-sulate are also called cryoprotectants. The wall material used in food products or processes should be food grade and must be able to form a barrier between the active agent and its surroundings (Zuidam and Nedovic, 2010).

Different types of encapsulates (reservoir, matrix and coated matrix) might be characterized (Figure 1). The reservoir type has a layer around the core material (also called capsule). The matrix type has the active agent dispersed over the carrier material and can be also found on the surface. A combination of these two types gives a third encapsulate called coated matrix, in which the active agent is a capsule covered by an additional layer (Lakkis, 2007).

Electron microscopy techniques are useful to obtain information on size range of bacteria-loaded and empty microcapsules, matrix microstructure and any matrix changes caused by the entrapped bacteria. By using cold stage scanning electron microscopy (cryo-SEM), it is possible to study the structure, configuration, and size distribution of capsules, and also differentiate capsules with or without bacteria. Cryo-SEM, including freeze-fracture, allows observing details of the matrix and the interaction between the carrier material and the bacteria. It is also possible to observe empty spaces around the bacteria. Transmission electron microscope (TEM) could be used to study the matrix microstructure, detect subtle changes both in bacteria and in the matrix, and also provide a more detailed view of the differences in the porosity of capsules with or without bacteria (Allan-Wojtas et al., 2008).

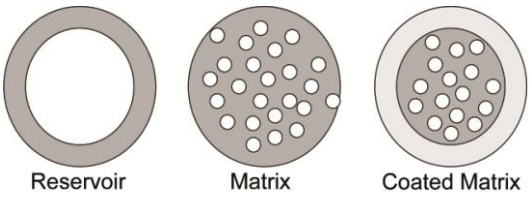

Figure 1. Types of encapsulates (Adapted from Zuidam and Nedovic, 2010).

MATERIALS FOR ENCAPSULATION

Carrier materials should serve as protection for probiotics and also be safe for consumption, that is, Generally Recognized As Safe (GRAS) and cost effective, since a high cost will directly influence the value of the final product. Low cost carrier materials include starches, inulin, pectin and most carbohydrates (De Vos et al., 2010). Other materials such as alginate and trehalose are often used but at a higher cost. Main materials for encapsulation are discussed below.

Polysaccharides

Agar, sodium alginate, carrageenan, gum arabic, chito-san, dextrans, starch and cellulose (ethyl-cellulose, acetyl-cellulose, methyl-cellulose, carboxymethyl-cellu-lose, nitrocellulose) are the principal carrier materials used for encapsulation. Sodium alginate is the most com-monly used material, compatible with almost all encap-sulation methods, and usually used in combination with other components (Burgain et al., 2011). It is a linear, unbranched amorphous copolymer composed of β-D-manuronic acid (M) and α-L-guluronic acid (G) linked by 1 → 4 bonds. The M and G units on alginates can be randomly arranged or organized as heterogeneous or homogeneous sequences. The chemical composition and distribution of a sodium alginate sequence depends on the species and parts of algae used in extraction (Fu et al., 2011).

Sodium alginate is widely used as a gelling agent, due to its ability to form hydrogels with divalent cations, such

as Ca^{+2}, Ba^{+2} o Sr^{+2} under moderate conditions. The hydrogel is formed because the blocks of guluronic acid bond with cations, resulting in a three-dimensional network of alginate filaments that are held together with ionic interactions. The model that best describes this network is the "egg-box model" (Simpson et al., 2004). Sodium alginate at a concentration of 20 g/L in combination with milk whey protein at 10 g/L has been used to encapsulate *L. plantarum 299v, L. plantarum 800* and *L. plantarum CIP A159* by freeze-drying (lyophilization).

Results indicate that encapsulated strains incubated in the gastric juice have greater viability than free bacteria and are able to survive the intestinal environment (Gbassi et al., 2010). Using chitosan at a concentration of 1% w/v to encapsulate extruded strains of *Lactobacillus acidophilus* 547, *Bifidobacterium bifidum* ATCC 1994, and *L. casei* 01, resulted in greater viability for *L. acidophilus* and best protection *L. casei* cells. No carrier material was able to keep *B. bifidum* viability because of its low resistance to acidic pH (Krasaekoopt, et al., 2004).

A mixture of alginate and modified starch are used to encapsulate the probiotics *L. acidophilus* and *B. lactis* by emulsion to incorporate them into yogurt. Encapsulated probiotics showed greater viability in storage as compared to free cells. A sensory analysis found that the use of encapsulated cells in yogurt did not alter color, acidity or flavor properties (Kailasapathy, 2006).

Cellulose acetate phthalate is used as a carrier material to prepare microcapsules with *L. acidophilus* (La-05) and *B. lactis* (Bb-12) by spray drying. Studying the resistance of microorganisms at dry temperature and tolerance to a simulated environment with pH and bile salts that are similar to those in the human stomach and intestine, Favaro-Trindade and Grosso (2002) found no reduction in the viability of *B. lactis* with an inlet temperature of 130°C and outlet of 75°C in the spray dryer. For *L. acidophilus*, a two log cycle reduction in viability was observed. After 2 h of incubation in solutions with pH 1 and 2, the microcapsules were effective in protecting the microorganisms and similar results were obtained with bile salt solutions (Favaro-Trindade and Grosso, 2002).

Oligosaccharides

Corn syrup, sucrose and maltodextrin are commonly used. When an appropriate mix of maltodextrin and trehalose was used as carrier media to encapsulate *L. paracasei* by spray cooling or freezing, high bacterial viability was obtained. The concentration of trehalose helped increase cell viability due to its high osmotic pressure (Semyonov et al., 2010).

Lipids

Waxes, paraffin, diglycerides, monoglycerides, fats, stearic acid, triestearins and oils are mainly used. When sesame oil was used to encapsulate *Lactobacillus delbrueckii subsp. bulgaricus* by emulsion, 5.4% increase in cells viability was observed under refrigeration conditions vs. free cells. Better cell viability of the encapsulated bacteria under simulated intestinal conditions was also observed (Hou et al., 2003).

Proteins

Gluten, casein, whey protein, albumin, are commonly used protein-based encapsulation materials. Picot and Lacroix (2004) worked with milk whey protein as a carrier material to encapsulate *Bifidobacterium breve* R070 and *Bifidobacterium longum* R023 by spray drying to be later included into yogurt. The results indicated that whey protein can increase the tolerance of the bacteria to acidic pH, therefore whey proteins have a great potential to be used in probiotics and in products where bacteria need to be viable when reaching the gastrointestinal tract (Picot and Lacroix, 2004). Also, when whey proteins were used to encapsulate *L. rhamnosus* by extrusion in a simulated gastrointestinal environment, the protein capsules formed a matrix providing protection to *L. rhamnosus* in acidic pH and produced an efficient controlled release of biomolecules with a subsequent absorption *in situ* at the specific destination (Doherty et al., 2011). Encapsulation materials and methods used to encapsulate LAB are presented in Table 1.

Encapsulation techniques: Operating parameters, advantages and disadvantages

Microencapsulation technologies were developed and applied successfully to protect probiotic bacteria (Table 1) from damage caused by external factors such as drying, packaging and storage conditions (e.g., time, temperature, moisture and oxygen), and the degradation in the gastrointestinal tract, especially due to extreme pH (2.5 to 3.5) of gastric juices and bile salts (Kailasapathy, 2006).
The selection of the encapsulation method depends on the required particle average size, the physical and chemical properties of the carrier material, the applications of the encapsulated material, the required release mechanism and cost. These parameters need to be studied for each specific organism and process (Parra-Huertas, 2010; Burgain et al., 2011).

Encapsulation methods

Spray drying

Spray drying is an appropriate technique for industrial applications on a large scale. A liquid mixture is atomized

Table 1. Encapsulation materials and methods used to encapsulate lactic acid bacteria (LAB).

Lactic acid bacteria	Encapsulation technique	Encapsulation material	Reference
Lactobacillus rhamnosus GG	Extrusion	Whey protein	Doherty et al., 2011.
Lactobacillus acidophilus 547, Bifidobacterium bifidum ATCC 1994 y Lactobacillus casei 01,	Extrusion	Chitosan, poly-L-lysine, sodium alginate.	Krasaekoopt et al., 2004
Lactobacillus acidophilus Bifidobacterium lactis (Bb-12)	Spray drying	Cellulose acetate phthalate	Favaro-Trindade and Grosso, 2002
Bifidobacterium breve R070 y *Bifidobacterium longum* R023	Spray drying	Whey protein	Picot and Lacroix, 2004.
Lactobacillus paracasei	Spray freeze-drying	Maltodextrin and trehalose	Semyonov et al., 2010
L. plantarum 299v, *L. plantarum 800 y L. plantarum* CIP A159	Lyophilization	Sodium alginate - whey protein	Gbassi et al., 2010
Lactobacillus acidophilus y *Bifidobacterium lactis*	Emulsion	Sodium alginate and modified starch	Kailasapathy, 2006
Lactobacillus delbrueckii ssp. bulgaricus.	Emulsion	Sesame oil	Hou et al., 2003
Lactobacillus casei NCDC-298	Emulsion	Sodium alginate – soybean oil	Mandal et al., 2006.
L. rhamnosus GG y *L. acidophilus* NCFM	Countercurrent spray	Sodium alginate - chitosan	Sohail et al., 2011
Lactobacillus paracasei NFBC	Spray drying	Acacia gum – reconstituted skim milk	Desmond et al., 2002
Bifidobacterium PL1	Spray drying	Starch	O'Riordan et al., 2001
Bifidobacterium infantis	Emulsion- Spray drying	Canola oil - sodium caseinate - fructooligosaccharide -dehydrated glucose syrup	Crittenden et al., 2006
Lactobacillus bulgaricus, Lactobacillus plantarum, Lactobacillus rhamnosus, Enterococcus durans, Enterococcus faecalis	Lyophilization	Skim milk – sorbitol - monosodium glutamate	Carvalho et al., 2003
Carnobacterium divergens, Lactobacillus salivarius y Lactobacillus sakei	Spray drying	Skim milk powder	Silva et al., 2002
Lactobacillus reuteri C10	Lyophilization	Skim milk - sucrose-lactose	Khoramnia et al., 2011
Lactobacillus F19 *Bifidobacterium* Bb12	Emulsion - Lyophilization	Sodium caseinate – transglutaminase - sunflower oil	Heidebach et al., 2010

in a tank using a nozzle or disk and the solvent is evaporated after coming into contact with hot air or gas (Yañez et al., 2002) (Figure 2). Its application in *Lactobacillus* spp. (Desmond et al., 2002) and *Bifidobacterium* (O'Riordan et al., 2001) received considerable interest.

The process parameters to be considered include:

1. Air flow configuration: co-current or counter-current flow
2. Strain type and its pre-adaptation to the carrier material (Corcoran et al., 2004)
3. Carrier material: low viscosity for easy flow, tasteless flavor and high solubility (Vega and Roos, 2006)
4. Drying temperature: the number of viable probiotics linearly decreases with outlet air temperature. The output optimum air temperature should be as low as possible and the intake air temperature must be in the range of 150 to 170°C. Slow feed rate allows temperatures close to 80°C
5. Drying time: a short drying time improves the probiotics viability
6. Storage conditions: the survival of probiotics is optimal with low water activity (<0.25) (Zuidam and Nedovic, 2010) and low temperature (4°C). These conditions were used in yogurt and are usually recommended for good stability of dried cultures throughout storage (Picot and Lacroix, 2004). Temperatures between 19 and 25°C (O'Riordan et al., 2001) were used to assess the viability of encapsulated bacteria maintained under adverse environmental conditions.

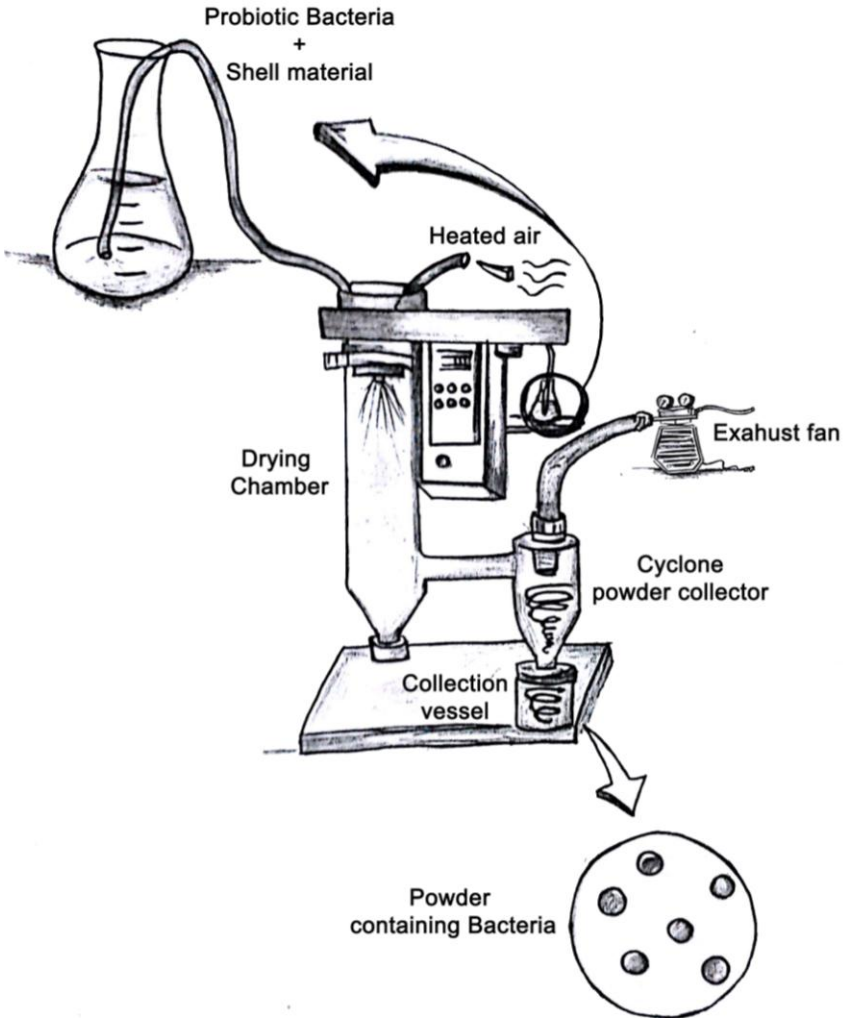

Figure 2. Schematic diagram of the spray-drying encapsulation method.

Spray drying has been used to encapsulate *L. paracasei* NFBC using gum arabic and reconstituted skim milk as carrier materials. Intake air at a constant temperature of 170°C was used. The culture was sprayed inside the drying chamber using a nozzle for two fluids, allowing the product to be dried almost instantly with very short residence time. Outlet temperature varied between 95 and 105°C. The capsules of *L. paracasei* NFBC prepared at temperatures between 95 and 105°C exhibited a 3 log increase in cell viability vs. free cells under the same conditions (Desmond et al., 2002).

Crittenden et al. (2006) obtained small capsules of *B. infantis* (15 to 20 µm in diameter) with a low water activity (0.2-0.3), using an oil in water emulsion prepared with canola oil, caseinate, fructooligosaccharide and dehydrated glucose syrup or starch resistant to microfluidization, as carrier materials. The inlet and outlet air temperature were 160 and 65°C, respectively. Also, the viability of the bacteria at storage room conditions (25°C) and the ability of the carrier material to protect *B. infantis* in a simulated human stomach and small intestine environments were studied. Microencapsulation significantly protected the bacteria at room temperature and in a simulated stomach and small intestine conditions when compared with no encapsulated bacteria (Crittenden et al., 2006).

Lyophilization

Lyophilization is done by freezing the probiotic together with the carrier material (typically between -30 and -20°C), followed by vacuum sublimation of water at absolute pressure between 0.05 to 0.1 mBar and temperature between -50 to -30°C. Once lyophilized, cryoprotectants are added to preserve and stabilize the probiotic activity

during storage. The most common cryoprotectans are lactose, trehalose, sorbitol, sucrose, milk protein and skim milk (Semyonov et al., 2010).

Encapsulated probiotics by lyophilization have better storage stability, especially at low temperatures and inert atmosphere (nitrogen or vacuum) (Zuidam and Nedovic, 2010). Unfortunately, lyophilization is 4 to 7 times more expensive than spray drying (Chavez and Ledeboer, 2007).

Carvalho et al. (2003) studied the effect of sorbitol and monosodium glutamate in solution with 11% skim milk on the storage viability of lyophilized *L. bulgaricus*, *L. plantarum*, *L. rhamnosus*, *Enterococcus durans*, *Enterococcus faecalis*. They reported a strong protective effect of sorbitol on the survival of the studied bacteria during storage. However, no significant differences in cell viability during lyophilization were observed. Monosodium glutamate after lyophilization showed an increase on LAB survival during storage. Consequently, the effect of each protective agent on the viability of a specific strain during or after the lyophilization process should be determined in a case by case basis.

Khoramnia et al. (2011) used response surface methodology (RSM) with central composite designs (CCD), to study the effect of cryoprotectants (skim milk, sucrose, and lactose) on the survival rate of the probiotic strain *Lactobacillus reuteri* C10 during lyophilization and storage for direct application in poultry. *L. reuteri* C10 has been characterized as having several probiotic properties. The central points used in the design were 12.5% (w/v) skim milk, 8% (w/v) sucrose, and 12.5% (w/v) lactose. The results showed that the presence of different combinations of cryoprotectants reduced the loss of cell viability during lyophilization. The loss of viability ranged from 0.26 to 0.66 log cfu/mL, while without cryoprotectants, values of 1.65 log cfu/mL were observed. The optimal combination of cryoprotectants for the presservation and storage of *L. reuteri* C10 was obtained with 19.5% skim milk, 1% sucrose and 9% of lactose. The survival rate of lyophilized *L. reuteri* C10 using the best combination of cryoprotectants and stored at 4 and 30°C for 6 months, was 96.4 and 73.8%, respectively.

Jin et al. (1998) using a supplemented diet with *Lactobacillus* lyophilized cultures to feed broiler chickens found that the presence of *Lactobacillus* cultures significantly increased body weight and feed : gain ratio of broilers during 0 to 6 weeks. RSM was used by Huang et al. (2006) to improve the viability of *L. delbrueckii subsp. bulgaricus* LB14 using sucrose, glycerol, sorbitol and skim milk during lyophilization. The results showed that the RSM not only helped in finding the optimal protective agent concentrations to maximize bacteria viability, but also provided adequate information to assess main effects and interaction among protective agents on cell viability.

Extrusion

The oldest and most common technique to produce capsules with hydrocolloids (e.g., alginate and carrageenan) consists of preparing a hydrocolloid solution, adding microorganisms and forming droplets by extruding the suspension through a syringe needle (laboratory scale) or a extruder (pilot scale) to free-fall into a hardening solution (e.g., calcium chloride) (Figure 3).

The size and shape of the formed pearl depend on the diameter of the nozzle and the distance between the nozzle and the $CaCl_2$ solution. This method is simple and cost effective. It does not cause cell damage and results in high cell viability (Krasaekoopt et al., 2003). The technology does not use harmful solvents and can be done under both, aerobic and anaerobic conditions. The main disadvantage of this method is that it is difficult to use in large scale production due to the slow formation of microspheres.

The survival of the probiotic microorganisms *L. acidophilus* 547, *B. bifidum* ATCC 1994, and *L. casei* 01 microencapsulated in chitosan-coated alginate pearls was evaluated in yogurt made with UHT milk and conventional pasteurization during storage at 4°C for 4 weeks. Sodium alginate (20 gL^{-1}) and chitosan (4 gL^{-1}) were used to prepare the pearls. The results showed that the survival of the encapsulated probiotic bacteria was greater vs. free cells in approximately 1 log cycle. During storage, the number of probiotic bacteria, with the exception of *B. bifidum*, remained above 10^7 cfu g^{-1}, minimum recommended to ensure a therapeutic effect. The *B. bifidum* count fell below 10^7 cfu g^{-1} after 2 weeks of storage. The UHT treatment in yogurt did not alter the probiotic bacteria viability when compared with conventional thermal treatment (Krasaekoopt et al., 2006).

Soto et al. (2011) studied the use of macro-capsules from *L. casei* DSPV 318 T, a probiotic inoculum from bovine origin, in two formulations: sodium alginate (10 g/L) and sodium alginate (5 g/L) + corn starch (5 g/L). These mixtures were dispersed in containers of 1 and 2 mL, frozen at -20°C, immersed in boiling water and then placed in a $CaCl_2$ (0.1 M) solution to promote polymerization and maintain the capsules shape and size. The capsules were stored at 18 and 4°C and their viability was recorded for 63 days. The results showed that the refrigerated capsules had greater viability *vs.* the capsules kept at room temperature. It was concluded that probiotics had a shelf life of at least 2 months and could be used as culture initiators in calves.

The balance of the intestinal ecosystem may be negatively altered by stress situations, the use of antibiotics or by feeding calves with milk substitutes instead of colostrum. These practices can cause morbidity and mortality of young calves and economic losses. The regular administration of a probiotic inoculum of bovine origin can

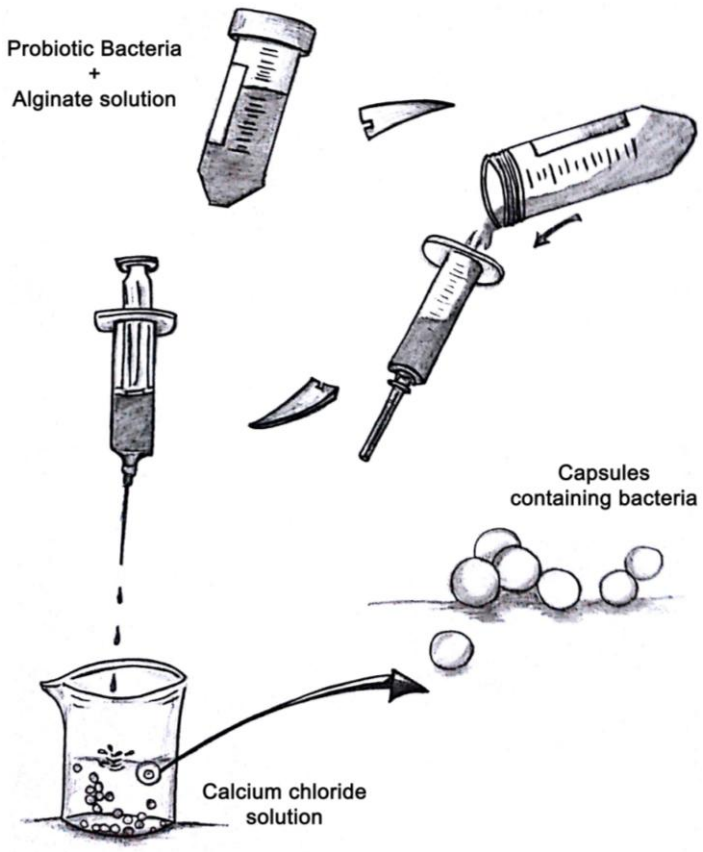

Figure 3. Schematic diagram of the extrusion encapsulation method.

promote a stable and balanced intestinal microbiota, and improve the calf's health (Soto et al., 2011).

Emulsion

This technique adds a small volume of a hydrocolloid suspension containing micro-organisms (discontinuous phase) to a large volume of vegetable oil (continuous phase). The mixture is homogenized to form water in oil emulsions by using an emulsifier. Once the emulsion is formed, it can be insolubilized to form gel capsules in the oil phase (Figure 4). The main disadvantage of this method is that it yields a wide range in particles size and shape (Burgain et al., 2011).

This technique has been used to encapsulate *L. casei* NCDC-298 in a matrix of sodium alginate, using soy oil as the continuous phase. 20 mL of alginate solution and 4 mL of cell suspension were mixed with 100 mL of soybean oil and 0.2% Tween 80, under continuous stirring. To hardened capsules and fragment the emulsion, 100 mL of 0.1 M calcium chloride were added. The results showed that encapsulation of *L. casei* NCDC-298 in sodium alginate, improved the viability of the bacteria in simulated intestinal conditions and under thermal treatment (Mandal et al., 2006).

This emulsion technique is relatively new in the food industry and easy to scale up. It provides encapsulated and trapped core materials. The particle size formed by this method is smaller (25 µm - 2 mm) than the size produced by the extrusion method (2 to 5 mm). Pearl size formed by extrusion depends on size of the needle, while particle size produced by emulsification depends on speed of agitation and type of emulsifier. The need for vegetable oil in the formulation may increase operation costs when compared with the extrusion method (Krasaekoopt et al., 2003).

Spray cooling or freezing (spray freeze-drying)

This procedure combines common steps used in lyophilization (freeze-drying) and spray drying, and has the advantages of providing controlled size and higher specific surface area vs. spray-dried capsules. However, the method also has disadvantages including high-energy requirement, long processing time and 30-50 times higher cost vs. spray drying (Burgain et al., 2011).

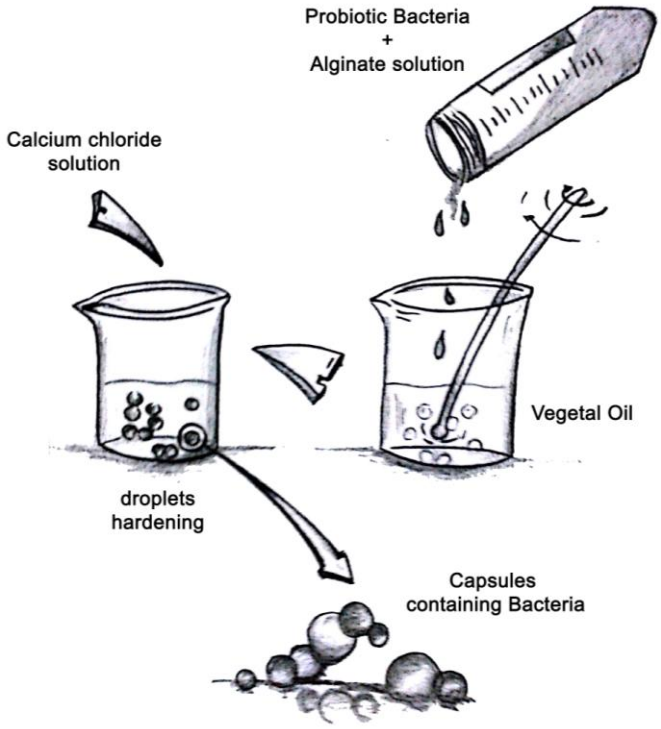

Figure 4. Schematic diagram of the emulsion encapsulation method (water-in-oil emulsion).

Table 2. Summary of encapsulation methods.

Encapsulation Method	Mechanism	Cost	Easy to Implement	Type of particle	Particle size (μm)
Spray drying	Dehydration	Low	Yes	Approximately spherical powders	3-100
Lyophilization	Sublimation drying	High	No	Irregular shapes	-
Extrusion	Reticulation	Low	Yes	Spherical capsules	1600-5000
Emulsion	Emulsification	Low	Yes	Spherical capsules	25-2000
Spray Freeze-Drying	Lyophilization-Spray drying	High	No	Approximately spherical powders	400-1400

Semyonov et al. (2011) encapsulated *L. paracasei* by spray freeze-drying using maltodextrin and trehalose as carrier materials. The technique consisted of spraying droplets containing a *L. paracasei* solution directly on liquid nitrogen and dehydrating the frozen droplets by lyophilization. Better bacteria viability was observed when using this technique than when using only lyophilization.

Another technique is based on aerosols of alginate and $CaCl_2$ solutions flowing from opposite directions in a chamber (Sohail et al., 2011). The technique consists of mixing a liquid bacteria culture with 2% sodium alginate solution, and then pumping the solution in the upper part of a plexiglass cylinder and in 0.1 M $CaCl_2$ solution in the bottom. Alginate solution droplets come in contact with $CaCl_2$ and fall to the bottom of the chamber from where the microcapsules are collected. Sohail et al. (2011) encapsulated *L. rhamnosus* GG and *L. acidophilus* NCFM using this technique and reported lower capsule sizes than those obtained by the traditional extrusion method. In addition, the micro-capsules were coated with chitosan, increasing the viability to simulated intestinal conditions. A summary of the encapsulation methods is presented in Table 2.

Use of enzymes in the encapsulation process

Transglutaminase enzymes have been used in probiotics encapsulation. The process is based on enzymatic gel

formation of the encapsulating material, and the subsequent application of one of the conventional encapsulation technologies. Heidebach et al. (2009) encapsulated a probiotic cell mixture with suspension of casein treated with transglutaminase and then applied the emulsion technique. This process provided 70 and 90% cell viability. The obtained capsules protected *Lactobacillus* F19 and *Bifidobacterium* Bb12 from pH damage levels simulating human stomach (Heidebach et al., 2009). These authors encapsulated *Lactobacillus* F19 and *Bifidobacterium* Bb12 with a water in oil emulsion and transglutaminase gel formation, and studied the effect of lyophilization and storage. Encapsulation was done using 15% (w/w) sodium caseinate as carrier material which was mixed with microorganisms and 10 international units of transglutaminase per gram of sodium caseinate at 40°C. Sunflower oil was finally added. The microcapsules were then centrifuged to separate the residual oil, lyophilized and stored at 4 and 25°C for 90 days. The results showed that the encapsulated *Bifidobacterium* Bb12 dropped *ca.* one log cycle in viability and the capsules showed an average of 3.8×10^9 cfu g^{-1} when stored at 4°C for 90 days. In *Lactobacillus* F19, the reduction was almost 2 log cycles with approximately 1.7×10^8 cfu g^{-1} at the end of storage time. The capsules contained the minimum required probiotic concentration (10^6 - 10^7 cfu g^{-1}) to have a therapeutic effect.

Stability of encapsulated materials

To determine the stability of encapsulated materials during storage, the glass transition temperature (Tg) measured needs to be established using differential scanning calorimetry (DSC) (Ndoye et al., 2007). The encapsulated product exhibits long shelf life when stored below glass transition temperature since deterioration due to bacterial proliferation and chemical reactions is very small. The low permeability of the carrier material at glass transition temperature is useful in preventing entrance of oxygen and preserving core materials. If storage temperature is set higher than glass transition temperature, various chemical reactions are accelerated because of the increase in the internal mobility of reagents and diffusion of oxygen (Qv et al., 2011).

Methods for controlling the release of encapsulated ingredients

Encapsulated probiotics need to be released from food products at the desired time and place. Understanding the chemistry of the carrier material allows controlling the release at a specific pH, temperature, and/or salt concentration. The release of the microcapsules must occur after crossing the gastrointestinal tract, releasing viable and metabolically active bacteria (Picot and Lacroix, 2004). Water-insoluble microparticles can increase their tolerance to high acidic environments favoring the release of probiotic cultures to the gastrointestinal tract (Ding and Shah, 2007). Thermal, physical and the dissolution method are the most common release mechanisms.

In the thermal release mechanism, the encapsulating protector material melts at a certain temperature, usually releasing the ingredient during cooking. By altering the type of carrier material and its thickness, it is possible to assure the release of the ingredient within a few degrees of the target temperature. Physical release occurs by physical breaking of microcapsules. This mechanism is generally designed for ingredients that should be released during chewing. Factors to be considered are capsule size, strength and flexibility of coating. In the dissolution method, the majority of food products contain at least a small amount of water, which can be used to assure the release of an ingredient trapped in a water-soluble coating membrane (Lakkis, 2007).

RESEARCH IN ENCAPSULATION

The additional cost associated with probiotic encapsulation must be fully justified in terms of offering a clear performance improvement. Since cost is an important competitive factor, the search for low cost encapsulating materials that enable protection and proper micro-organism release is required.

Carrier materials that protect LAB and offer other benefits including functional, nutraceutical and prebiotic properties, should be studied (Crittenden et al., 2006).

In addition, there are natural compounds that provide specific functional properties, such as the *Aloe vera* gel, which has *in vivo* and *in vitro* immune modulatory activities (Reynolds and Dweck, 1999) of interest in the food industry (Martínez-Romero et al., 2006). This gel inhibits the growth of several pathogens, including *Staphylococcus aureus* and *Streptococcus agalactiae* (Reynolds and Dweck, 1999). It has been used for encapsulation by freeze-drying (Serna-Cock et al., 2012) with promising results as a material to encapsulate LAB, since it preserves the viability of *Weissella confusa* (83.3% survival rate) when compared with free cells. It also yielded higher viability (80%), when compared with cells encapsulated in a sodium caseinate (10%) solution.

A new encapsulation method based on the use of supercritical fluid technology has been proposed by Moolman et al. (2006). These authors used an inter-polymer complex formation in supercritical carbon dioxide. The method was used to encapsulate indomethacin and *Bifidobacterium longum* in a poly (vinyl pyrrolidone) – poly (vinyl acetate–co-crotonic acid) interpolymer complex. The encapsulation matrix was stable at low pH, but

was disintegrated at higher pH, triggering release of the encapsulated material. Interpolymer complex encapsulation showed potential for protection of probiotics and therefore for application in the food and pharmaceutical industries (Thantsha et al., 2009).

Aro et al. (2013) used oat polar lipids produced by supercritical fluid technologies in the encapsulation of probiotics. The protective effects of the oat polar lipids were evaluated by measuring the gas production, microbial activity, acetic and lactic acid production, and pH changes in different test mediums. The results demonstrated that the polar lipids from oats were able to suspend probiotic bacteria in such way that they stay viable after being put into frozen storage and then re-suspended in aqueous systems.

In vivo studies are necessary to understand cell viability and capsule stability in real environments. In a research conducted by Kanmani et al. (2011), *in vivo* test were performed using six fasting albino male Wistar rats. Each rat was fed orally with microcapsules made by extrusion containing the probiotic strain *Enterococcus faecium* MC13. After feeding, the rats were sacrificed at 2 h intervals until 12 h. The stomach and intestine of each rat were extracted and washed with sterile water to observe the microcapsules with a microscope. Results showed that up to 4 h, stability and uniformity in size were observed in the recovered microcapsules; after 6 h, capsules began to break and the carrier material (alginate) provided a direct and easy delivery of probiotic cells in the intestinal region.

Similarly, the minimum physical and chemical properties that make a material promising for encapsulation of biological compounds (probiotics) remain unknown. More research to set specific properties, such as viscosity, molecular weight, gel formation, composition and glass transition temperature, is needed for specific applications.

Conclusion

Given the number of variables involved in optimizing an encapsulation process for a bioactive molecule (for example, encapsulation material, encapsulation technique, release mechanism and processing conditions), tools including response surface, genetic algorithms and sequential quadratic programming should be used. The main stages in the optimization include diagnostic experiments that involve theoretically promising variables for the encapsulated material, proposing the encapsulation according to the experimental design, construction of response surface models, optimization model formulation improvement of optimization and verification of optimal conditions. Research focused on physical and chemical properties of encapsulation materials, *in vivo* tests for release mechanisms, encapsulation of low-cost materials that comply with probiotic and prebiotic functions, is needed. It is essential to maintain stability, viability and cellular concentration during storage to ensure product efficiency for applications in food industry.

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Review

Ecology of pulpal and periapical flora

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Inflammatory lesions in the periapical tissues of the teeth are a result of root canal infection following partial or complete breakdown of the pulp. Infected root canals have a complex microbial flora consisting of cocci, rods, spirochaetes, filaments and fungi which may exist as a loose collection in mist canal lumen or as dense aggregates (biofilms) adhered to dentinal walls. The biofilm environment has been found to be advantageous for bacterial living; as it helps the bacteria to survive and multiply, inducing their metabolic products that will lead in the persistence of periapical infection.

Key words: Inflammatory lesions, periapical tissues, root canal infection, bacterial.

ETIOLOGY OF PERIAPICAL DISEASE

The essential role of microorganisms in the pathogenesis of periapical lesions was demonstrated by Kakehashi et al. (1965). They made experimental pulp exposures by drilling a hole through the occlusal thickness of enamel and dentin of maxillary first molars in germ-free and conventional rats, they found that the absence or presence of a microbial flora is the major determinant of healing versus development of periapical lesions. In the conventional animals, the exposed pulps became necrotic, and periapical granulomas or abscesses occurred in all cases. However, in the germ-free rats, the exposed pulps healed with dentinal bridging while no necrotic pulps, apical granulomas or abscess developed in spite of gross food impaction.

In agreement with this was the findings Möller et al. (1981). They severed the pulps of teeth in monkeys. The amputated pulps were either immediately sealed aseptically or left open to be contaminated with indigenous oral flora for 1 week and then sealed. After 6 to 7 months, clinical, radiographic and histologic examinations of the teeth that were sealed aseptically showed no pathologic changes in their periradicular tissues. In contrast, teeth with infected root canals had inflammatory reactions in their tissues. The previous findings confirmed that bacteria are the primary etiologic factor in the development and progression of pulp and periapical diseases.

ASSOCIATION BETWEEN ROOT CANAL BACTERIA AND PERIAPICAL LESION DEVELOPMENT

There is a range of periapical responses to the root canal microbial flora that can occur. These responses may include acute periapical inflammation, chronic periapical inflammation, chronic suppurative periapical inflammation, acute periapical abscess/cellulitis, periapical osteomyelitis, periapical osteosclerosis or condensing osteitis, granulomas epithelial proliferation and cysts.

Korzen et al. (1974) studied the effects of normal oral flora and mono-infection (*Streptococcus mutans*) on the pulp and periradicular tissues of conventional and gnotobiotic rats, its results showed that the severity of pulpal and periradicular inflammation was directly related to the quantity of microorganisms. Furthermore, it showed that the degree of inflammation was less severe with monoinfection than with mixed infection. Until the early 1970s, most microbiological studies on root canal flora reported primarily the presence of facultative bacteria in this

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system. However, technologic advances in the isolation of anaerobes and increased awareness of the medical and dental progressions of the role of anaerobes in various diseases caused significant changes in medical and dental microbiology. Sundqvist et al. (1985) showed that root canal infections are multibacterial and that anaerobic organisms, namely *Bacteroides* species, play a significant role in clinical signs and symptoms of pulpal and periradicular disease. *Bacteroides* species have undergone classification changes. New genus names, *Porphyromonas* and *Provotella*, have been assigned to many of the *Bacteroides* organisms (Siqueira and Rocas, 2005).

Other investigators (Fabricius, 1982; Fabricius et al., 1982a, b) in a series of experiments, examined the importance of bacteria in the development of periradicular lesions, composition of root canal flora, and the influence of a combination of oral bacteria on oral periradicular tissues of monkeys. In one study, the researchers mechanically devitalized the pulps of monkeys, left them exposed to oral flora for 1 week, and then sealed them for 3, 6, and 35 months. Bacteriologic examinations of infected root canals at the end of these observation periods showed that 85 to 98% of the isolated bacteria were anaerobic. The most frequently found anaerobic species were *Bacteroides* and Gram positive anaerobic rods. A small percentage of facultative anaerobic bacteria were also isolated from the infected root canals. In another study, they inoculated 75 root canals of monkeys with 11 bacterial species separately, or in combinations, and sealed the access cavities for a period of 6 months. Their bacteriologic and histologic examinations showed that mixed infections have a greater capacity to cause apical lesions than do mono-infections. Furthermore, they reported that the *Bacteroides* strain did not survive in the root canals when inoculated as pure cultures, and facultative streptococci induced small periradicular lesions. To account for the possible contribution of unsampled or uncultivated bacteria in the pathogenesis of lesions, eleven isolated strains (including eight strains from one tooth, representing its total cultivable infection) from previous studies were inoculated in freshly necrotized monkey teeth, in various combinations, but always in equal proportions. The "eight-strain collection" consisted of *Bacteroides oralis, Fusobacterium, necrophorum, Fusobacterium nucleatum, Streptococcus milleri, Streptococcus feacalis (Enterococcus feacalis), Peptostreptococcus anaerobius, Actinomyces bovis* and *Propionibacterium acnes.* After six months, the "eight-strain collection" was recovered from all teeth, and interestingly, in the same proportions that it had been recovered from the original tooth (Dahlén et al., 1987). This suggested that selective pressures were at play in the root canal system that reproduced the "same infection". Other combinations did not survive effectively; some species were not recovered at all. Sundqvist et al. (1985) demonstrated a high correlation between the presence of *Prevotella melaninogenica* and clinical and radiographic signs and symptoms of periradicular pathosis. Griffee et al. (1980) also found a similar correlation between the presence of this organism and pain, sinus tract formation and foul odour. Other researchers (Yoshida et al., 1987) found that *Peptococcus magnus* and *Bacteroides* species were commonly associated with symptomatic cases. Haapasalo (1989) reported on the bacteriology of 62 infected human root canals, giving special attention to the *Bacteroides* species. His results confirmed the findings of previous investigations: almost all root canal infections are mixed, and acute symptoms are usually related to the presence of specific anaerobes, such as *Porphyromonas (Bacteroides) gingivalis, Porphyromonas (Bacteroides) endodontalis* and *Prevotella (Bacteroides) buccae*.

Brook et al. (1991) confirmed the polymicrobial nature of bacteria isolated from aspirates of periradicular abscesses in 39 patients, with anaerobic isolates being present in more than 70% of the bacteria recovered. Wasfy et al. (1992) obtained similar results in the microbiologic evaluation of periradicular infections when they found that anaerobic bacteria were the predominant flora in specimen cultures. Anearobes comprised 73% (190 of 259) of cultivable bacteria. Using molecular technique, Wang et al. (2010) investigated the occurrence of *P. gingivalis* fimA genotypes and its possible correlation with the clinical symptoms, from 158 infected root canals with apical periodontitis. Their findings showed that *P. gingivalis* was detected in 39.9% of the infected root canal samples and was found in 44.5% of *P. gingivalis* positive specimens with symptoms. Types II (69.4%) were the most frequent in the symptomatic cases followed by type IV (32.7%). The occurrence of type I (64.3%) was significantly higher than any other genotypes in the asymptomatic apical periodontitis, whereas types II and Ib were not identified. Statistical analysis revealed that the occurrences of types II, IV, and Ib fimA were associated with greater risk of clinical signs (swelling, sinus tract, or intracanal exudates) than type I.

It can be concluded from the previous findings that root canal infections are associated with great bacterial diversity and high correlation exist between the composition of the microbiota and the clinical signs and symptoms.

Nature of root canal flora

The microorganisms in nature are presented in two forms: in aqueous based environment called planktonic or found in aggregation or communities with polysaccharide matrix called biofilm. Biofilm is defined as thin layer condensations of microbes (e.g. bacteria, fungi and protozoa) that may occur on various surfaces in nature. Such films may become established on any organic or inorganic surface substrate where planktonic microorganisms prevail in water-based solution. Organisms in biofilms were shown to have stronger pathogenic potential

than those in planktonic state (Chavez de Paz et al., 2007; Siqueira et al., 2007; Svensater and Bergenholtz, 2004; Mah and O`Toole, 2001). It was proven that the biofilm bacteria are up to 1,000 times more resistant than planktonic bacteria to phagocytosis, antibodies, antibiotics, disinfectants and antimicrobials (Chai et al., 2007).

The presence of biofilm in the endodontic infections was initially reported by Nair in 1987. He reported a well condensed bacterial layer on the dentinal wall of the root canal which when visible in light microscopically, gave the palisade structure of bacterial plaques adhering to tooth surfaces. It was proven in many studies that the presence of biofilm was associated with persistent and chronic periapical periodontitis. Carr et al. (2009) examined resected root tip of failing endodontically re-treated lower molar tooth under light and electron microscope. They found complex, viable, multispecies biofilm in the entire length of the specimen. Using SEM, several studies were able to detect bacterial biofilm near the apical foramen of teeth that were resistant to endodontic treatment and with necrotic pulps or associated with chronic periapical periodontitis (Tronstad et al., 1990; Molven et al., 1991; Noiri et al., 2002; Leonardo et al., 2002).

Siqueira and Lopes (2001) examined 26 extracted teeth diagnosed as having asymptomatic periradicular lesions and were associated with extensive caries and periapical pathosis. The SEM photographs showed cocci and rods restricted to the root canal, and only one case showed dense bacterial aggregates close to the apical foramen. Clinical isolates of spore-forming gram positive aerobic rods from three patients with persistent periapical periodontitis were identified as *Bacillus subtilis* that exhibited dense meshwork-like structures in their cell surfaces. It was suggested that *B. subtilis* could form bio-films in periapical periodontitis lesions, and this might contribute to resistance to treatment in those patients (Yamane et al., 2009). Likewise, *Actinomyces* sp. oral and *Propionibacterium* were shown to be important contributors to extraradicular biofilm formation and persistent periapical infection (Wang et al., 2012)**.**

Histological examination of the root canal treated teeth with apical periodontitis was correlated with the findings of clinical observations. The results showed heavier intraradicular bacterial colonization (biofilm) associated with apical root canals of both untreated and treated teeth with apical periodontitis (Ricucci et al., 2009; Ricucci and Siqueira, 2010). In conclusion, the biofilm was proven to form in the infected root canal system as well as in the external root surface especially in cases with persistent periapical pathosis and this could be correlated to the resistance of such cases to conventional endodontic treatment and hence contributed to the treatment failure.

Flora of root canal and periradicular lesions

Current concepts suggest that the number of bacterial species in an infected root canals may vary from one to more than 12 and the number of bacterial cells from $<10^2$ to $>10^8$ per sample (Sundqvist, 1992).

Early studies (Winkler and van Amerongen, 1959) generally reported a predominance of facultative organisms over obligate anaerobic species. *Streptococcus*, Gram-negative cocci, and *lactobacilli* were most often recovered, usually found in numbers that constituted less than 50% of the total isolates reported. Through the use of improved techniques, a large variety of bacteria (genera and species) have been isolated from root canals and periradicular lesions. Other studies have found that the organisms most often found appear to be normal flora of the oral cavity; only rarely is a bacterium recovered that can be shown to originate from other parts of the body (Le Goff et al., 1997; Munson et al., 2002; Sundqvist, 1976). The composition of the microbiota from different infected root canals shows a great variability (Love, 2009; Montagner et al., 2012)

There is now a consensus of opinion that the root canal flora of non-carious teeth with necrotic pulp and diseased periapex is dominated (>90%) by obligate anaerobes (Bystrom and Sundqvist, 1981; Haapasalo, 1989; Sundqvist, 1976; Sundqvist et al., 1989) usually belonging to the genera *Fusobacterium*, *Porphyromonas*, *Eubacterium* and *Peptostreptococcus*. On the other hand, the microbial composition, even in the apical third of the root canal of periapically affected teeth with carious crowns, is less dominated (<70%) by strict anaerobes (Baumgartner and Falkler, 1991).

In general, a mixed flora of bacteria has been isolated in the various studies. Epidemiologic studies have shown that more than 200 different microbial species can be found in infected root canals, usually in combinations of 4 to 7 species per canal (Baumgartner and Falkler, 1991; Munson et al., 2002; Sundqvist, 1992; Sundqvist, 1994, Gomes et al., 2013). Theoretically, any one of these species would have the potential to be an endodontic pathogen. Studies (Haapasalo, 1989; Sundqvist et al., 1985) have suggested that several *Bacteroides* species are more likely to be involved rather than just one species. Obviously, the organisms that do eventually become involved in root canal infections have to survive a harsh selection process. Although finding several different species at once is common, it is usual for one or two of the species to dominate the mixture.

Debelian et al. (1992) recovered *Propionibacterium acnes* from root canals and blood samples taken during and after patient treatment. *Streptococci mutans* was reported to be non-cariogenic in mono-infected gnotobiotic rats by Watts and Paterson (1992) but the same organism was associated with extensive periradicular inflamemation 28 days after the creation of untreated pulpal exposures.

Sato et al. (1993) investigated the bacterial composition of necrotic pulps of human teeth by sampling the split surfaces of freshly extracted teeth and culturing for microorganisms using reliable anaerobic techniques. Of the

276 bacterial isolates, 251 (91%) were obligate anaerobes. The genera *Peptostreptococccus* (25%), *Propionibacterium* (19%), *Eubacterium* (17%) and *Fusobacterium* (13%) were most commonly recovered. *Bifidobacterium* (2%), *Lactobacillus* (1%), *Actinomyces* (1 %) and *Veillonella* (0.7%) were also recovered. The microflora of necrotic pulps of human deciduous teeth was, in the authors' conclusions, similar to that reported for the deep layers of dentinal lesions of adults.

Studies recognizing synergy or a positive correlation between species are also available. Simonson et al. (1992) reported a highly significant synergistic relationship between *Treponemadenticola* and *Porphyromonas (Bacteroides) gingivalis*, whereas Sundqvist (Sundqvist, 1992) found strong positive correlations between *Fusobacterium nucleatum* and *Peptostreptococcus micros* and *Porphyromonas (Bacteroides) endodontalis*, *Selenoomonas sputigena* and *Wolinella recta*, *E. feacalis* and *Fusobaccterium nucleatum*. Recently, Ribeiro et al. (2011) investigated the bacterial diversity in untreated asymptomatic teeth (n = 12) exhibiting periapical lesions, by 16S ribosomal-RNA (rRNA) sequence analysis. They demonstrated, that despite being highly diverse, the microbiota of primary endodontic infections is mostly represented by members of the phylum *Firmicutes* belonging to the class *Clostridia* followed by the phylum *Bacteroidetes.* Using checkerboard DNA-DNA hybridization, Sassone et al. (2012) showed differences between the composition of the microbiota in cases with exposed and unexposed pulp space. The species found in higher counts in exposed pulp space cases were *Eubacterium saburreum, Fusobacterium nucleatum* ssp. *vincentii, Tannerella forsythia, Enterococcus faecalis, Neisseria mucosa, Campylobacter gracilis* and *Prevotella nigrescens*, while in unexposed pulp space cases they were *F. nucleatum* sp. *vincentii, N. mucosa, E. faecalis, E. saburreum, C. gracilis,* and *P. gingivalis, F. nucleatum* sp. *vincentii, Campylobacter sputigena, Capnocytophagashowae, Treponemasocrenskii, Porphyromonas endodontalis, Eikenellacorrodens and Capnocytophagaochracea*.

In addition, unidentified spirochetes have been found in necrotic root canals using microbiological methods, darkfield and transmission microscopy, and molecular techniques (Brown and Rudolph, 1957; Dahle et al., 1993; Hampp, 1957; Kantz and Henry, 1974; Nair et al., 1990, 2005; Nair, 1997; Thilo et al., 1986).

Recent studies have revealed a possible role for fungi and viruses in endodontic infections (Baumgartner et al., 2000, Gomes et al., 2010). The incidence of yeasts cultured from primary endodontic infections varies from 0.5-61.5% (Akdeniz et al., 2002; Goldman and Pearson, 1969) depending on the culturing method. The most common species isolated were *Candida albicans*, and less frequently *Candida sake* and *Rodotorula mucilaginosa* (Egan et al., 2002). The occurrence of herpes viruses in periapical lesions has been investigated (Sabeti et al., 2003; Hernadi et al., 2013). Herpes simplex virus infection demonstrated no relationship with periapical disease. Periapical lesions harboring cytomegalovirus / Epstein-Barr virus dual infection tended to show elevated occurrence of anaerobic bacteria, which is symptomatic, and exhibit large size radiographic bone destruction (Sabeti and Slots, 2004).

DISTRIBUTION OF ENDODONTIC MICROORGANISMS

It is evident that bacteria can inhabit not only the main root canal, but also enter the dentin tubules, apical canal ramifications, isthmuses and other morphological irregularities of the root. A number of studies have shown that invasion of bacteria into dentin tubules occurs in 60-90% of teeth with apical periodontitis (Matsuo et al., 2003; Peters et al., 2001). There are also suggestions that bacteria found in the dentin tubules are special and unique in comparison with the microflora of the oral cavity. Existing knowledge about the ability of different species to invade dentin shows that such species as Gram-positive facultative *cocci*, *lactobacilli* and *Actinomyces* are more often found as invaders among other bacteria species. Obviously, the environment of the tubules restricts supply of nutrients to bacterial species making their life conditions less favorable (Matsuo et al., 2003; Peciuliene et al., 2008; Peters et al., 2001).

These bacteria may reach and colonize the most apical part of the root canal and thereby be in close contact with the periradicular tissues through the apical foramen and accessory foramina. Watts and Paterson (1992) found bacteria in only a minority of sections of root canals and periradicular tissues of albino rats, with and without traumatic pulpal exposures. Walton and Ardjmand (1992) found bacterial masses at the apical foramen of induced periradicular lesions in monkeys and concluded that such masses could contaminate periradicular tissues during surgery or extraction and could give a false-positive result on microbiologic sampling.

Bacteria infecting the apical region of infected root canals are predominantly anaerobic, and this dominance seems to be directly proportional to the time of infection. In a study in monkeys, Fabricius et al. (1982a, b) aimed to evaluate the distribution of different microbial species in root canal samples after different periods of time and in different parts of the root canal system. The relative proportions of anaerobic bacteria cells increased with time and facultative bacterial cells were outnumbered when the canals were infected for 90 days or more. After 90 or 180 days of infection, 85 to 98% of the bacterial cells in root-canal samples from the apical region were anaerobic. Baumgartner and Falkler (1991) cultured the apical of 5 mm of root canals of 10 teeth with carious exposures and reported that the most prevalent species were *Prevotella intermedia/nigrescens*, *Prevotella buccae*, *Peptostreptococcus anaerobius*, and *Veillonella parvula*, all of them being isolated from half of the examined cases.

Of a total of 50 bacterial isolates, 68% were strict anaerobes, demonstrating the predominance of such organisms in this site. On the other hand, Chugal et al. (2011) investigated the bacterial communities residing in the apical portion of human teeth with apical periodontitis in primary and secondary infections, using a culture-independent molecular biology approach. They demonstrated that the apical bacterial communities in primary infections were significantly more diverse than in secondary infections and sequencing findings revealed a high prevalence of *Fusobacteria, Actinomyces species* and oral *Anaeroglobus geminatus* in both types of infection. Mean time, secondary infections contained *Burkholderiales* or *Pseudomonas* species, both of which represent opportunistic environmental pathogens.

Dougherty et al. (1998) investigated the occurrence of black-pigmented anaerobic bacteria in the apical and coronal segments of infected root canals and found these bacteria in 12 of 18 cases (67%). *Prevotella nigrescens* was isolated from 9 of 12 apical segments, *Prevotella melaninogenica* from 3 of 12, *P. intermedia* from 1 of 12, and *P. gingivalis* from 1 of 12. Siqueira et al. (2004) investigated the prevalence of 11 selected putative endodontic pathogens in the apical third of infected root canals associated with periradicular lesions using a nested polymerase chain reaction (PCR) assay. Their results showed *Pseuramibacteralactolyticus* in 6 (26%), *Fusobacterium nucleatum* in 6 (26%), *Porphyromonas endodontalis* in 4 (17%), *Filifactor alocis* in 2 (9%), *Dialister pneumosintes* in 1 (4%), *P. gingivalis* in 1 (4%) and *Tannerella forsythensis* in 1 (4%). No sample yielded *P. intermedia*, *P. nigrescens*, or *Campylobacter rectus*. Of the samples examined, 17 were positive for at least 1 of the target species. Occurrence of these bacterial species in the apical third of infected root canals suggests that they can be involved in causation of periradicular lesions (Siqueira et al., 2004). Similar bacterial taxa were also demonstrated in the apical root canal system of seventeen extracted teeth with attached apical periodontitis lesions (Rocas et al., 2010). The most prevalent taxa in the apical root canal system were *Olsenellauli* (76.5%), *Prevotella baroniae* (71%), *P. endodontalis* (65%), *Fusobacterium nucleatum* (53%) and *T. forsythia* (47%). While *Streptococcus* species were more prevalent in middle/coronal samples.

Therefore, it can be concluded that bacterial profile differs according to the type of endodontic infections (primary versus secondary) and the level of the root canal system (coronal/middle/apical).

BACTERIAL SAMPLING AND COLLECTION METHODS

Precise identification of microorganisms participating in the pathogenesis of apical periodontitis is important in order to understand the disease process and to provide effective antimicrobial treatment. Traditionally, endodontic bacteria have been studied by means of cultivation-based techniques, which rely on isolation, growth and laboratory identification, by morphology and biochemical tests. For a long time, culturing and serial dilution methods were considered as standard methods used in research (Peciuliene et al., 2008). However, these methods have been demonstrated to have several limitations when it comes to microbiological diagnosis (Relman and Falkow, 1992). During the past decade, the analysis of endodontic microbiota experienced a shift from culture-based to molecular approaches. Nevertheless, it is important to differentiate between the two methods, culturing measures viable bacterial cells as colony-forming units while molecular methods measure nucleotide sequences and viable microorganisms are not required. The molecular method allows amplification of very minute quantities of DNA to detectable levels. High-throughput DNA sequencing methods have provided a deeper understanding of the oral microbiota and identified putative endodontic pathogens that were not previously found with culture-based methods. In endodontics, pyrosequencing has been used to elucidate the bacterial diversity in necrotic root canals. Li et al. (2010) demonstrated that a 600-foldincrease in the depth of coverage can be obtained with pyrosequencing when compared with traditional Sanger capillary sequencing. Siqueira et al. (2011) revealed a high degree of bacterial diversity in resected root tips of necrotic teeth by using pyrosequencing. Ozok et al. (2012) identified 606 taxa (species or higher taxon) in infected root canals, representing 25 microbial phyla or divisions. Whereas, Saber et al. (2012) identified 35,731 high-quality DNA sequences belonging to 10 bacterial phyla and 73 bacterial genera from seven symptomatic periapical lesions.

TREATMENT OF PERIAPICAL DISEASE

The elimination of endodontic infection is different from elimination and control of most other infections in the human body. Because of the special anatomic environment in the root canal and tooth, host measures that are sufficient to eliminate the infectious organisms in other sites do not suffice for complete elimination of endodontic infections. Therefore, control of an endodontic infection is a concerted effort by several host and treatment factors. Success in all aspects of this cooperation will eventually result in elimination of the infective microorganisms and healing of apical periodontitis. Studies have shown that when the root canals are properly instrumented, disinfected, and obturated, success rate of endodontic treatment was approximately 80-90% for teeth with periapical periodontitis (Sunde et al., 2002). Although, chemo mechanical cleaning and shaping is effective in spread of infection and provide symptom relief in case of apical periodontitis, systemic antimicrobial therapy might be useful for preventing the spread of pathogens to other

anatomic sites in high-risk patients. *E. faecalis* should be the target of antibiotic therapy in cases of secondary infection, whereas streptococci and strict anaerobes should be targeted in cases of primary infection (Skucaite et al., 2010).

Studies have shown that bacterial infection can be substantially reduced by standard intracanal procedures, however, it is difficult to render the canal free of bacteria as it may survive and recolonize the root canal space and this may become a focal source of persistent infection (Chivatxaranukul et al., 2008; Rocas and Siqueira, 2010, 2011). In addition, the complex anatomy of root canal system including accessory canals, grooves, and isthmuses will not allow the direct access of disinfectants during bio-mechanical preparation. Therefore, supplementary ap-proaches to reduce the load of the intra-canal microbes were suggested. These include mechanical agitation of the irrigant solution, photodynamic therapy and cold plasma therapy (Balto, 2008; Al-Madi and Balto, 2008; Ng et al., 2011; Silva et al., 2012; Pan et al., 2013).

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Destruction of *Giardia Lamblia* by electrical treatment of infected tab water

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Protozoan parasites are responsible for the majority of gastrointestinal infections and even drinking treated tap water was reported risk factor for sporadic giardiasis. Variety of technologies has been used for elimination of *Giardia* cysts as well as removing other microbial contaminants and particles. The cysts are usually identified by their indistinguishable morphology. We applied direct current (DC) electrical charges for 1 to 4 min, 10 mA or 12 V, and 15 mA or 18 V in a handmade electrolytic cell filled with a prepared 10^5 cysts per ml emulsion, to examine its effectiveness as cysticide. Fresh human stools with *Giardia*, stained with Eosin Gelblich, were used to prepare wet mounts. Samples were examined by conventional light microscopy for the presence of cysts. Temperature and pH of the emulsions were measured in all stages. DC electrical charges equal or more than 15 mA (18 V in our circuit), in 2 min are practically lethal for *Giardia* cysts found in human stools, in a watery base with this concentration. pH of the emulsions varied between 3.5 to 8.8 in 22-24.4°C, when the currents were applied. 5 min after closing the DC electrical circuit, the tank water was treated and could be used.

Key words: *Giardia lamblia*, water treatment, direct electrical currents, cysticides.

INTRODUCTION

Waterborne giardiasis has been reported in epidemic form among humans in many countries and drinking water is the most reported vehicle of spread. Swallowing water while swimming, eating lettuce and even drinking treated tap water are reported risk factors for sporadic giardiasis in the United Kingdom (Stuart et al., 2003). Intestinal protozoan parasites are responsible for the majority of gastrointestinal infections in subtropical areas (Ravdin, 1995) and about 58 million children suffer from giardiasis every year (Escobedo et al., 2009). Although higher incidence of intestinal parasites in lower socio-economic societies has been reported as a health inequities but spreading of giardiasis caused by drinking water is also reported in several states of United State (Espelage et al., 2010).

Environmental occurrence of *Giardia* has been reported in variety of sources. Jakubowski and his colleagues examined raw sewage waste water in 11 cities of Unites State and reported a correlation between raw sewage cyst level and reported cases of giardiasis (Jakubowski et al., 1991). Mayer and Palmer (1996) examined samples from a large metropolitan wastewater plant in California. They reported 13000 cyst/L in influent and 11 cyst/L in secondary effluent samples.

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Cysts have also been found in surface water, drinking water, groundwaters, cisterns, soil, surfaces and foods in North America and United State (Hancock et al., 1997; Crabtree et al., 1996; Cornell University Report, 1993; Cody et al., 1994 and Fayer et al., 1998). Viable *giardia* cysts are highly infective for humans. In a controlled clinical study of volunteers who were fed *giardia* cysts contained in conventional gelatin capsules, eight dosage levels ranging from 1 to 1,000,000 cysts per capsule have been examined. A single dosage of ten cysts were found to be clinically infectious in human (Rendtorff, 1979).

Results of the latest (2005) National Research Project in Iran showed that the prevalence of gastrointestinal parasitic infections has been about 19.3% while 10.9% of the patients suffered from *Giardia Lamblia* (Sayyari et al., 2005). Lack of water filtration as well as inadequate personal hygiene, socio-economic status, poverty, lack of sanitation and geographic factors were mentioned as the main reasons causing parasitic infections in Tehran, the capital of Iran (Shojaei Arani. 2008).

Giardia genuses are binucleate, flagellated protozoan parasites which attach to the wall of the human small intestine. *G. duodenalis*, *G. Intestinalis* or *G. lamblia* are common variety of symptoms in human Giardiasis. *G.lamblia* pyriform bodies range from 9 to 21 µm wide and 2 to 4 µm thick. They are identified by their two morphologically indistinguishable anterior nuclei, eight flagella, two central axonemes, microtubular median bodies and a ventral adhesive disk (US. EPA report, 1999). *Giardia* could be transmitted via the fecal-oral route of exposure. Giardiasis can be an asymptomatic infection in humans but it may cause acute or chronic diarrhea, steatorrhea, abdominal cramps, bloating, flatulence, weight loss and vomiting (Hall. 1994).

Because of the resistance of *Giardia* cysts to environmental conditions, high prevalence and low infectious dose in humans, variety of water treatment techniques have been used to remove or inactivate *Giardia*.

Most frequent physically removal technologies to reduce water turbidity by removing microbial contaminants and particles are: conventional/direct/membranes such as microfiltration, ultra filtration, nanofiltration, and reverse osmosis/ slow sand/ diatomaceous earth (DE) filtration methods. They almost remove 99.9% of *Giardia* cysts when operated under appropriate coagulation conditions (US. EPA report, 1999). However, Ongerth (1990) found poor removal of *Giardia* cysts as the major deficiencies in the operation of three small water plants with either conventional filtration, direct filtration, or DE filtration. Because possible outbreaks occurred in filtered water systems, frequent sampling and monitoring of treatment effectiveness are necessary for effective removal of cysts. Inactivation of cysts up to 99% or more can be achieved by disinfectants although several factors including water temperature, pH, residual and applied disinfectant concentration, contact time; some particles which may shield cysts from contact may increase disinfectant demand. *Giardia* can be resistant to low dose of chlorine and chloramines and inactivation efficiencies of the various disinfectants are different. Jakubowski (1990) studied utilized water disinfectants effectiveness of inactivation of the cysts and sorted them in the following decreasing order: ozone, mixed oxidants, chlorine dioxide, iodine, free chlorine, and chloramines.

Slavik (1993) used a low-current, low-voltage electrical treatment as a means to either kill *Giardia lamblia* or prevent excystation of this protozoan.

Karanis et al. (1998) examined sensitivity of protozoan to Ultraviolet (UV) irradiation and found that reduction of *Giardia* cysts requires a dose of 1800 J/m2. The comercial UV units irradiate much less doses and are not reliable. The new generation of powerful UV devices showed capabilities to reduce to less than 90% of *Giardia* cysts at the maximum dose tested.

MATERIALS AND METHODS

In an experimental study, with a pilot scale study design with four constant repetitions, we examined the effectiveness of electrical direct currents (DC) as a cysticide for control of *Giardia* where treated tab water is not available. Fresh stools were used to prepare wet mounts that were examined by conventional light microscopy for the presence of cysts (Figure 2). Samples counted positive for *Giardia* based on their distinctive morphology.

Tab water was boiled for 30 min and kept in a sterile reservoir and cooled down to room temperature (23-25°C). Its pH was controlled before and after boiling. Four samples were collected in the University Reference Lab from the Giardiasis patients diagnosed by finding *G. lamblia* and cysts with their feces. In each case, 1 ml of the fresh stool sample was added to the boiled water to prepare diluted emulsion. The emulsions then passed through 2 layers of sterile filter pads to clear up and centrifuged at 900 *g* for 1 min. The sedimented layers were diluted again to reduce sample turbidity. The samples were monitored by staining with Eosin Gelblich dye solution for detection of cysts. 0.1 ml of sample was mixed to 1 ml of prepared dye and stained cysts were mounted on hemocytometer counting chamber of the Light microscope using an oxford sampler pipette. Based on this counting method, 4 containers of the emulsion with 10^5 cysts per ml was prepared. Temperature and pH of the emulsions were controlled before electrical interventions. 10 ml of controlled emulsion were decanted by a pipette in a handmade, 10 CC, semi-cylindrical electrolytic cell which had two platinum plates (electrodes) at the two ends of the main slot (Figure 1).

Duration of the DC electrical treatments were 1 to 4 min and two low currents of 10 mA or 12 V, and 15 mA or 18 V (in our electrolytic cell), were applied at each of the four durations. Temperature and pH of the emulsions were measured inside the tank, soon after each particular dosage of currents applied.

In the next step, the whole content of the tank was poured in the test tube for further samplings. Samples were also taken from the electrode's surface by swab sticks. After similar staining, all samples were mounted on hemocytometer counting chamber to be exactly monitored by the same rater. The same process was repeated 96 times in different durations, intensities applied to the four stool samples and three times repetition.

Electrolytic cell was washed and carefully cleaned using alcohol, sterile water and cotton at each repetition. The wall of the tank and the surface of both electrodes were checked after each washing by

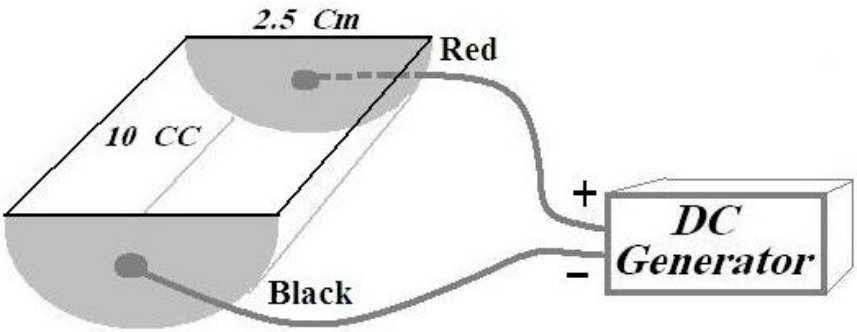

Figure 1. The electrolytic cell used to apply DC electrical charges; handmade by the means of two semi-circular Platinum plates and a semi-cylindrical tube.

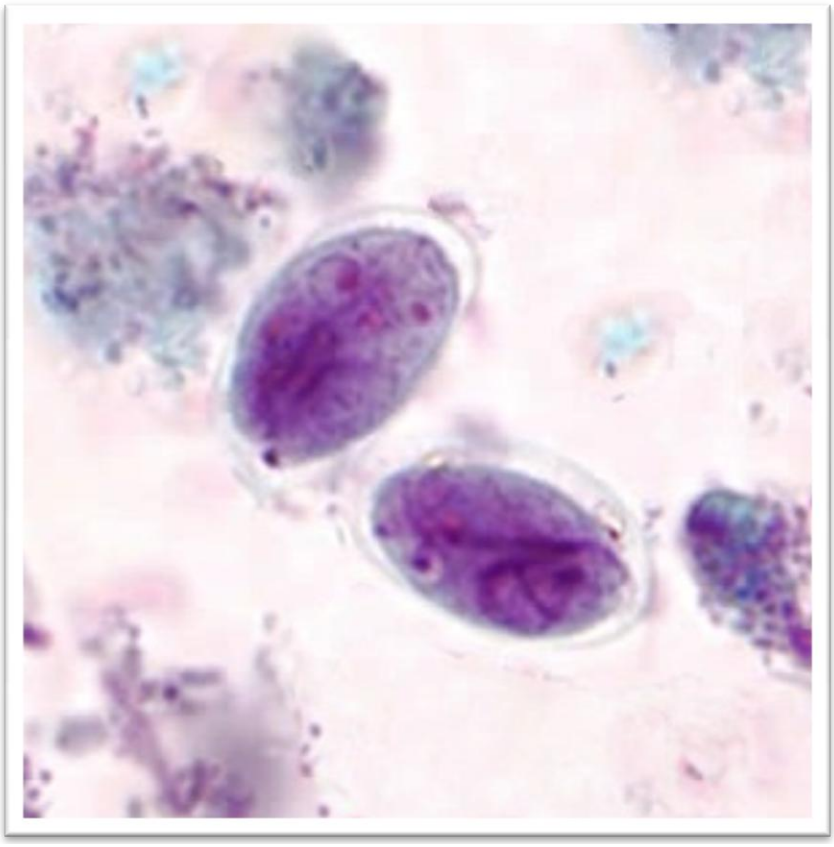

Figure 2. *Giardia lamblia* cysts stained with Eosin Gelblich.

three separate wet swab samplings.

We used a programmable electrical current generator to apply the electrical currents with pre-set parameters (3646A – DC power supply, Circuit Specialists Inc. USA). The conventional hand controlled generators could not be used for such short time current durations with the constant level of intensity since there is no distinction between impedance and resistance in DC electrical circuits. To calculate the exact current intensities at different sites of electrolytic cell, we measured the Ohmic resistance of each part of the tank by an impedance meter (ZM-104A, TOA, Japan). In each set of samples and before opening the electrical current, electrical resistances of the four following parts of the circuit were measured: 1) The whole circuit [from the tip of Anode wire [+] to the tip of Cathode wire (-), Red wire to Black wire tips]; 2) Inside the filled tank (from mid-point of each wall, inside the emulsion); 3) Cathode resistance [from cathodic platinum plate (black side) to the middle of the tank]; anodic resistance [from anodic platinum plate (red side) to the middle of the tank].

Having the electrical resistances, the intensity of the currents were calculated based on the Ohm law; V = R x I, where V is the voltage of DC current on the generator device (volts), R is measured resistance (ohms) and I is the intensity of current (Amperes).

Table 1. Effects of direct electrical currents in various intensities and durations on concentration of the 10^5 cyst/ml emulsions.

Current	Time (min)				
	0	1	2	3	4
(mA)-(V)	**Cyst Concentration** - per ml				
10-12	10^5	10^5	8 x 10^4	5 x 10^4	2 x10^4
15-18	10^5	10^4	0	0	0

Table 2. Effects of direct electrical currents in various intensities and durations on the pH and the temperature inside the tank. When the electric current is open,variable pH of electrode sides noticed.

Time (min)	Applied currents (mA)			
	10	15	10	15
	Temperature (°C)		pH	
0	22	22	7.2	7.2
1	22.5	23	4.5 – 8.2	3.7 – 8.7
2	22.8	24	4 – 8.3	3.5 – 8.8
3	23	24.4	3.9 – 8.5	3.4 – 8.8
4	24	24.4	3.7 – 8.7	3.2 – 8.8

RESULTS

As the main finding of the study, *Giardia* cysts found in human stools, in a watery base with the concentration of 10^5 cysts/ml could be practically counted up to 0 whenever DC currents equal or more than 15 mA (18 V in our circuit) are applied for 2 min (Table 1).

The measured electrical resistances were: 0.5 KΩ close to the anode electrode, 0.4 KΩ close to the cathode electrode, 1 KΩ inside the tank, and 1.2 KΩ for the whole circuit. Therefore, for the 18 V current, the current intensities which caused the complete cysticide effects should be: 36 mA close to the anode electrode, 45 mA close to the cathode electrode, 18 mA inside the tank, and 15 mA for the whole circuit.

Tap water pH did not change after 30 min boiling but the emulsions pH changed considerably during DC electrical treatments. When the currents were opened, pH reached to the minimum amount of 3.5 close to the anode and maximum amount of 8.8 around the cathode. These changes reversed to the normal rates after 285 s (less than 5 min).

When an electrical current passes through a circuit with resistance, heat production is inevitably expected. In our electrolytic cell, maximum temperature enhancement after 4 min of DC electrical treatment was 2.4°C (Table 2).

DISCUSSION

Although viability determination might not be necessary for determining the effectiveness of the treatment process for physically removed or injured cysts, but it is very important in assessing disinfectant efficacies of treated water before drinking.

We used dye staining, morphological criteria and cyst counting as the post treatment procedures to determine the efficacy of each particular electrical intervention.

The discontinuity in gradual relationship between electrical current duration at its proper intensity as a cysticide was expected, since the lethal charge level of electricity for *G. lamblia* should have a sharp threshold instead of variable broad ranges.

As a result of polarization of electrodes and formation of H^+ ions around cathode and OH^- ions around anode, pH of electrolytic cell was variable after closing DC currents. These changes are unstable in watery environments and usually reverse when the ions combine and turn to water again (http://en.wikipedia.org, 2013).

The parameters we used in the electrolysis of *Giardia* cysts should be effective on cysts from human. The host source of the cysts may play some undefined roles. Hibler and Metzger (1974). also suggested this possibility. However, this speculation will require several sets of experimentations on variety of *Giardia* cysts from different sources to be approved or ignored.

Viability of cysts could be significantly affected as results of pH changes. Rubin et al. reported an average viability of 91% at pH 5, 56% at pH 7, and 62% at pH 9 all at 15° C, with a variability not exceeding ±5% (Rubin, 1989). The viability of cysts in our preparations was matched and count controlled to have emulsion with 10^5 cysts per ml. The emulsions pH was changed from 3.5 to 8.8 in 22 to 24.4°C when the currents were applied (Table 2).

Although both recent parameters may have influenced

the viability of Cysts but other factors, such as electric DC charge, should be responsible for the Zero viability (all killed). A positive correlation could be seen between the rate of applied DC voltage, intensity, duration and its efficacy to massacre the cysts.

The duration of the low current, low voltage of DC electric charges were shown in this study to be cysticidal for *Giardia* at room temperature and are similar to those suggested by Slavik et al. (1993).

Slavik found that electrical treatment can kill *Giardia* trophozoite in 5 min or less in both static and flow through systems. Electric treatment was also able to prevent excystation of *G. lamblia*. Hass and Aturalive (1999) also studied the effects of very short duration pulses of high voltage electrical current (Electroporation) on the viability of *Giardia* cysts, Cryptosporidium oocysts, and reported a minor effect on the cyst survival when the electrical pulses were used. In electroporation, usually short term, several hundred volts of alternative electrical current would be applied on the cell plasma membrane in molecular biology to change its electrical conductivity and permeability. Haas and Aturaliye (1999) used it in the presence of free chlorine, combined chlorine, hydrogen peroxide and potassium permanganate. They concluded that the combination of electroporation and those chemical disinfectants could produce superior inactivation of cysts and would be the treatment of choice to inactivate resistant protozoa in water. In our application of DC electricity, the only possible biological effects could be the enhancement, reduction or block of cyst's membrane caused by the bipolar DC charge of electricity somehow similar to the Iontophoresis of the skin.

The technique, if used in water treatment plant, would be safe without adding extra chemicals to drinking water. In DC electrical circuits, the only hazard could be the electrical shock. The perception of electric shock can be different depending on the voltage, duration, current, path taken, frequency, etc. Current entering the hand has a threshold of perception of about 5 to 10 mA for DC. The shocks can be felt only in sudden touch or in opening-closing of the current (http://www.electricityforum.com. 2013). Therefore, the current intensities used in this method could be at sensory threshold of perception.

Since the findings of the study on effectiveness of DC electrical treatment is based on results of laboratory experiments, additional studies should be conducted to compare the effectiveness of electricity under representative conditions in natural waters and local water plants.

Our results clearly demonstrate that viable *Giardia* cysts can be destroyed reliably by a short time DC electrical power and that this destruction can take place even in a few minutes using a simple electrolysis tank. We should emphasize that when electrical treatment is used as a cysticide against *Giardia,* attention should be given to the pH, ions, change after DC treatment and the tank water should be used at least 5 min after closing the electrical circuit.

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Comparison of soil microbial communities between high and low yield organically managed orchards

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Microbial communities play an important role in soil nutrient cycling. The present study aimed to evaluate the relationships of plant species, soil physical and chemical properties and microbial communities between high- and low-yield organically managed fruit orchards. The Biolog™ and phospholipid fatty acids (PLFAs) analysis were used to evaluate soil microbial communities for eight typical organic orchards in Beijing, China. These included high- and low-yield agricultural sites for four types of fruit orchards: pear, peach, apple and grape. The soil properties including soil organic matter (OM), soil pH, soil total nitrogen (TN), soil available phosphorus (AP), soil available K (AK), cation exchange capacity (CEC), soil bulk density, soil porosity, microbial biomass and microbial activities were investigated. There were significant higher microbial biomass and lower bulk density in the soils of high-yield orchards than that in low-yield orchards. Differences between the paired soils of high- and low-yield orchards were highly associated with the average well colour development and total PLFAs.

Key words: Microbial community, biolog, substrate utilisation, phospholipid fatty acid, organic agriculture.

INTRODUCTION

Organic agriculture is an ecological production management system that promotes and enhances soil biodiversity, biological cycles and biological activity. It is based on minimal use of off-farm inputs and on management practices that restore, maintain and enhance ecological harmony. The primary goal of organic agriculture is to optimise the health and productivity of interdependent communities of soil microbes, plants, animals and people. This method avoids the use of synthetic chemical fertilisers and genetically modified organisms to influence the growth of crops, and emphasises environmental protection, animal welfare, food quality and health, sustainable resource use and social justice objectives (Goewie, 2003). Organic agriculture gives priority to long-term ecological health, such as biodiversity and soil quality, rather than short-term productivity gains. In comparison, conventional farming utilises large quantities of inputs in the form of synthetic fertilisers, pesticide, labour and capital to improve food and fibre productivity to meet the current human demand, leading to a number of markedly undesi-rable side-effects such as environmental damage and degradation of several ecosystem services (Goewie, 2003). Problems arising from conventional practices have led to the development and promotion of organic farming systems that account the environment and public health as main concerns, and are recognised as environmental friendly production systems. In recent years, organic agriculture has gained worldwide acceptance and has deve-

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loped rapidly, at an annual rate of approximately 20% in the last decade and now accounting for >32.2 million ha worldwide.

Organic farming has been shown to improve many environmental and human components of the agro-ecosystem. However, it is clear that organic yields are usually lower, with the extent depending on the crop (Trewavas, 2004). So that organic agriculture cannot meet the increased demand for food and hence cannot be considered a sustainable form of farming for the future (Anifowoshe, 1990). The major challenge of organic farming systems is to maintain high yields and excellent quality by utilising farming practices that have acceptable in terms of environmental impacts (Murphy et al., 2007). There are relatively sudden heavy requirements for nitrate to produce leaf protein for chloroplasts and photosynthesis during plant growth and therefore maximal crop yields are likely only when the provision of soil nitrate and the associated crop requirements for leaf production are synchronised. This temporally uneven requirement for N in spring can only be matched by careful application of soluble chemical fertilisers. However, organic material is only slowly degraded over many months or even years and cannot release minerals in the short intense burst required for plant growth (Trewavas, 2004). Maintaining soil fertility is important for keeping high soil productivity in organic agriculture. Soil fertility in organic farming systems is based on the fixation of atmospheric nitrogen (N2), combined with recycling of nutrients via bulky organic materials, such as farmyard manure and crop residues, with only limited inputs of permitted fertilisers (Gosling and Shepherd, 2005; Nguyen et al., 1995). Plant nutriaents in organic farming are usually released slowly and are highly dependent on the mineralisation processes that make nutrients available to plants (van Delden, 2001).

The primary source of mineral nutrients for plants is the decomposition of organic matter by soil microbes (Hamilton and Frank, 2001). Microbial communities are responsible for a vast number of functions of agricultural soils. Microorganisms are key players in the mineralisation of N, phosphorus and sulphur, which are vitally important for plant nutrition and contribute significantly to the formation of soil aggregates (Esperschutz et al., 2007). Soil microbial biomass generally comprises about 2–3% of the total organic carbon in the soil, and is recognised as an important source of nutrients due to its fast turnover. A strong positive correlation has been found between the amount of nutrients held in the microbial biomass and the amount of mineralisable nutrients in soil (Marumoto et al., 1982), indicating that nutrient cycling is tightly linked to the turnover of microbial biomass. Soil microbial community and their activities, therefore, play a key role in maintaining soil fertility and should receive attention in studies on the direct effects of crop or soil management practices.

The fruit orchard production system can be considered as a complex and interacting system consisting of soil, plants and environment. Soil fertility is vital to crop yield, and is defined as the ability of a soil to provide the conditions required for plant growth. Soil microbial communities are important for the functioning of the ecosystem, both in relation to direct interactions with plants and with regard to nutrient and organic matter cycling. Soil microbial community is positive correlated with soil quality and soil fertility. Strategies to control the microbial community associated with plant growth systems need to be based on a fundamental understanding of the factors which structure and regulate the community (Garland, 1994).

In this paper, we attempted to evaluate the relationship among soil physical and chemical properties, fruit yields and soil microbial communities in high- and low-yield organic fruit orchards. Substrate utilisation assay was used to analyse the heterotrophic microbial communities (Garland and Mills, 1991; Buyer et al., 2002), and phospholipid fatty acids (PLFAs) analysis was used to examine the overall microbial community structure, independent of culturability of the microorganisms.

MATERIALS AND METHODS

Soil sample

The soils were originally sampled in eight organic orchards for planting apple (*Malus domestica*), pear (*Pyrus* sp.), peach (*Prunus persica*) and grape (*Vitis* sp.) at an agricultural experiment station of Beijing, in northeast China. These four fruits were the most important fruits grown within the temperate zonobiome of China. Locations of the samples were determined using a GPS. The soils were sampled from high- and low-yield of the four fruits orchards respecttively. All samples assayed in this report were collected using the same collection method. Soil samples were taken from the top 20 cm of the soil using a 2.5 cm diameter auger. Every single soil sample was taken from eight cores, then well mixed and sieved (2 mm mesh), and all samples were sub-sampled from these bulk soils. The sub-samples were immediately dispatched in cooled containers. The soil samples were refrigerated during preparation for analyses.

Chemical and physical analyses of soils

Soil samples were submitted to the Institute of Forestry and Pomology, Beijing Academy of Agriculture and Forestry Sciences for the following analyses: Soil organic matter (OM), soil pH, total soil nitrogen (TN), available soil phosphorus (AP), available soil K (AK), cation exchange capacity (CEC), soil bulk density and soil porosity. The soil properties were determined according to standard methods as follows: Soil pH was measured by a combination glass electrode (soil: water = 1:2.5). Total nitrogen was determined using Kjeldahl digestion method, and soil OM was determined using dichromate oxidation method. AP analysis was done using the method of Olsen and Sommers (1982), TN measured using alkaline hydrolysis diffusion method, and the available soil potassium (AK) was measured by the neutral ammonium acetate extraction method. CEC was measured by ammonium acetate method, and soil bulk density determined by cutting-ring method (Lu, 2000).

Soil microbial community analysis

Community level physiological profiling

Community-level physiological profiles (CLPP) were assessed using Biolog™ EcoPlate (Biolog, Hayward, California, USA). The

EcoPlate microplates were used to analyse substrate utilisation patterns of soil microbial communities in these experiments, and performed according to Campbell et al. (1997). Briefly, a 100 ml soil suspension was generated from an original 10 g aliquot (dry-weight equivalent) in 0.87% NaCl solution, thus achieving a 10^{-1} dilution. This solution was vigorously shaken for 10 min on a shaker at 200 rpm for 20 min. Ten-fold serial dilutions were made and 150 µl of the 10^{-3} dilution solutions of each soil sample were transferred into each well of a Biolog microtiter plate. The plates were incubated at 25°C in darkness and measured optical densities (OD) at 590 nm every 24 h for 7 d using an Emax precision microplate reader (Campbell et al., 1997). Raw OD data are corrected by blanking each response well against its own first reading (immediately after inoculation). Values of the respective control-well were subtracted. The negative values that occasionally resulted were set to zero. According to the Biolog data sheet, the net absorbances at 0 and 168 h were the data chosen for statistical analysis. The rate of average well colour development (AWCD) was calculated from each plate at each reading time. The Shannon diversity index (*H'*) was calculated from the following equation:

$$H' = -\sum_{i=1}^{s} pi \ln pi$$

$$Pi = (C - R)/\sum(C - R)$$

R is the OD of the control well, and *S* is the number of substrates, *pi* is the ratio of the corrected absorbance value of each well to the sum of absorbance value of all wells. Evenness (*E*) was calculated as: $E = H'/H_{max} = H'/\ln S$, where H_{max} is the maximum value of *H'*, and *S* is the number of substrates (Khalil et al., 2001).

PFLAs analysis

Microbial community structure was assessed by PLFAs analysis. Lipid extraction and PLFAs analysis were performed following the Frostegård method with minor modifications (Frostegård et al., 1993; Bååth and Anderson, 2003). Briefly, the soil was extracted in a single-phase mixture consisting of chloroform, methanol and citrate buffer (1:2:0.8 v/v/v). After splitting the extracts into two phases by adding chloroform and buffer, the lipid-containing phase was dried under a stream of Nitrogen. The lipid materials were fractionated on silicic acid column into neutral lipids, glycolipids and polar lipids (phospholipids). The phospholipids were transesterified by a mild-alkaline methanolysis and the resulting fatty acid methyl esters were analysed using a gas chromatography–mass spectroscopy (GC–MS) system (Hewlett Packard HP 6890) equipped with an HP-5 capillary column (60 m × 0.32 mm), and the mole fraction of each component was calculated. Methyl nonadecanoate fatty acid (19:0) was used as the internal standard. The fatty acid nomenclature chosen for this study was described by Frostegård et al. (1993). The sum of the following PLFAs was used a measure of the bacterial biomass: i14:0, i15:0, a15:0, 15:0, i16:0, 10Me16:0, i17:0, a17:0, cy17:0, 17:0, br18, 10Me17:0, 18:1ω7, 10Me18:0 and cy19:0 (Frostegård and Bååth, 1996). The PLFA 18:2ω6,9 was taken to indicate predominantly fungal biomass (Klamer and Bååth, 2004). Ratios of Gram-positive to Gram-negative bacteria were calculated by taking the sum of the predominant Gram-positive PLFAs 16:0(10Me), 17:0(10Me), 18:0(10Me), i15:0, a15:0, i16:0, i17:0, and a17:0 divided by the sum of the predominant Gram-negative PLFAs 16:1ω5, 16:1ω7t, 16:1ω9, cy17:0, 18:1ω5, 18:1ω7 and cy 19:0 (Yao et al., 2000). A ratio of the fungal/bacterial PLFAs was used as a biomass index to indicate the changes in the ratio of fungal to bacterial biomass. Total PLFAs concentration was used as an index of the total viable microbial biomass.

Statistical analysis

All ANOVA, regression and multivariate analyses were conducted using the statistical program SPSS 16.0. Means and least significant differences were calculated using one-way ANOVA.

RESULTS

Physical–chemical characteristics of soil samples

The land geographical location and physical–chemical characteristics of soil samples are shown in Table 1. Soil pH was weakly alkaline with range 7.55–8.49. The highest levels of available phosphorus (AP) and soil available (AK) were in the soil samples of the high-yield apple orchard: 261.0 and 202.0 mg/kg, respectively. The lowest AP (23.1 mg/kg) and the lowest AK (111.6 mg/kg) contents were in the low-yield and high-yield pear orchards, respectively. Soil OM, TN, AK and AP were higher in the high-yield orchards of apple and grape than in the pair site low-yield orchards. Whereas, in pear and peach orchards, the contents of soil TN, OM and AK were lower in high-yield than low-yield orchards. The soil bulk density in high-yield was lower than in low-yield orchards for the four types of fruit. The CEC in grape and peach orchards was higher in high-yield than low-yield orchards; in contrast, CEC was lower in apple and pear high-yield compared to low-yield orchards.

CLPP

Biolog carbon substrate metabolic activities

The carbon substrate metabolic activities of soils from the high-yield and low-yield orchards of four kinds of fruit were measured by AWCD (Figure 1), calculated from each sample's incubation time period. AWCD of Biolog EcoPlates is an important index for evaluating diversity of soil microbial biomass function. The values represent the changes in soil bacterial community activity in utilising the catabolic diversity in different treatments. AWCD increased rapidly after 48 h. AWCD in the soil of the high-yield were consistently higher than in low-yield orchards for the four types of fruit trees. This indicated that soil in the high-yield orchard had higher soil bacterial activity in utilising catabolic diversity. The soil microbial communities under the low-yield orchards used fewer carbon substrates and had lower metabolic activity than that of high-yield orchards. The OD data at 590 nm for six kinds of carbon sources indicated that soil microbial populations in different fruit orchard can vary tremendously.

The lowest utilisations for the metabolism of amines/amides, amino acids, carbohydrates, carboxylic acids, miscellaneous and polymers (Figure 2) were associated with the soil of the low-yield grape orchard. However, carbohydrates and miscellaneous had OD significantly greater in low-yield than high-yield grape orchards (Figure 2). For soil of peach orchards, the highest OD was in the amino acids utilisation of high-yield peach orchards. The utilisation of carbon substrates was higher

Table 1. Physical and chemical properties of soils, the standard deviation of the mean is given in brackets.

Soil type	Geographical location	Soil property						
		Soil bulk density (g/ml)	TN (g/kg)	SOM (g/kg)	AP (mg/kg)	AK (mg/kg)	CEC (mmol/kg)	Soil pH
High-yield apple	116.20°E 40.22°N	1.52 (0.10)	1.09 (0.06)	18.8 (1.31)	202.0 (33.15)	261.0 (23.64)	187 (8.19)	7.62 (0.47)
Low-yield apple		1.53 (0.08)	0.79 (0.34)	15.9 (5.45)	110.2 (40.55)	187.3 (42.25)	209 (6.08)	7.55 (0.15)
High-yield grape	116.67°E 39.92°N	1.10 (0.28)	1.38 (0.52)	22.9 (0.90)	73.5 (12.95)	190.7 (46.29)	211.3 (4.5)	8.00 (0.27)
Low-yield grape		1.57 (0.06)	0.99 (0.16)	18.2 (2.30)	81.6 (2.50)	133.0 (8.89)	181.7 (8.62)	8.36 (0.05)
High-yield pear	116.33°E 39.73°N	1.49 (0.02)	0.49 (0.10)	8.13 (1.42)	66.5 (31.01)	111.6 (13.76)	79.5 (10.11)	8.49 (0.19)
Low-yield pear		1.53 (0.05)	0.81 (0.06)	14.8 (1.35)	23.1 (12.54)	120. 7 (28.02)	165.0 (21.0)	8.21 (0.08)
High-yield peach	117.10°E 40.13°N	1.23 (0.06)	1.14 (0.08)	16.9 (1.82)	28.1 (22.44)	177.3 (27.30)	242.3 (13.32)	8.09 (0.13)
Low-yield peach		1.47 (0.01)	1.18 (0.21)	21.7 (2.05)	62.9 (13.60)	244.0 (29.5)	242 (17.1)	8.00 (0.03)

in high-yield than low-yield peach orchards, except for miscellaneous. For pear orchards, the utilisation of carbon substrates was higher in high-yield than low-yield orchards, except for polymers. For apple orchards, the utilisation of carbon substrates in high-yield was all higher than that in low-yield orchards.

Metabolic diversity

The diversity indexes were used to represent the level of the utilisation of carbon resources by the soil microbial community. The Shannon-Wiener index (H) and Simpson's diversity index suggested a diversity of carbon substrates were utilised by soil microbial communities in the orchards (Table 2). The soils exhibited slight differences between the high-yield and low-yield orchards. The Shannon-Wiener (H) and Simpson's diversity index were higher in high-yield than low-yield orchards, except for pear orchards. The evenness index had the same trend as the Shannon–Wiener index.

PLFAs analysis of microbial communities

The PLFAs are major cell membrane constituents. Different subsets of microbial community shown different PLFAs patterns, and since PLFAs could be degraded rapidly upon cell death, they were good indicators of living organisms.

We investigated the soil microbial communities in the soils of high- and low-yield orchards by analysing the composition of the PLFAs (Table 3). The total PLFAs content showed great variation among the fruit orchards, with range 9.3–47.6 nmol g^{-1}dry soil. The highest total PLFAs concentration was in the soils of the high-yield grape orchard (47.6 nmol g^{-1} dry soil); the second highest concentration was in the soils of the high-yield apple orchard (46.7 nmol g^{-1} dry soil). The lowest total PLFAs content was in soils of the low-yield peach orchard (9.3 nmol g^{-1} dry soil); the second lowest was in the low-yield pear orchard (18.8 nmol g^{-1} dry soil). The total PLFAs content in high-yield orchards was always significantly higher than the corresponding low-yield fruit orchards. There were differences in bacterial biomass (estimated by PLFAs content) between high-and low-yield orchards. The total bacterial PLFAs content was significantly higher in the high-yield than low-yield orchards of peach, apple and grape. Gram-positive bacteria showed the same trend as bacterial biomass, that is, more abundant in high-yield orchards of peach, grape and apple than in low-yield orchards; however, abundance was greater for the low-yield pear compared to the high-yield pear orchard. Gram-negative bacteria were more abundant in high-yield orchards of all fruit types compared to low-yield orchards. High-yield orchards of all fruit types had lower GP/GN ratios compared to the corresponding low-yield orchards. The amount of fungal PLFAs was higher in the high-yield than the low-yield orchards of the four types of fruit trees. The high-yield orchards had higher fungi/bacteria ratios than low-yield orchards of peach, pear and grape, but not for apples.

DISCUSSION

Soil is an important natural resource and medium for plant growth, its quality is crucial for sustaining

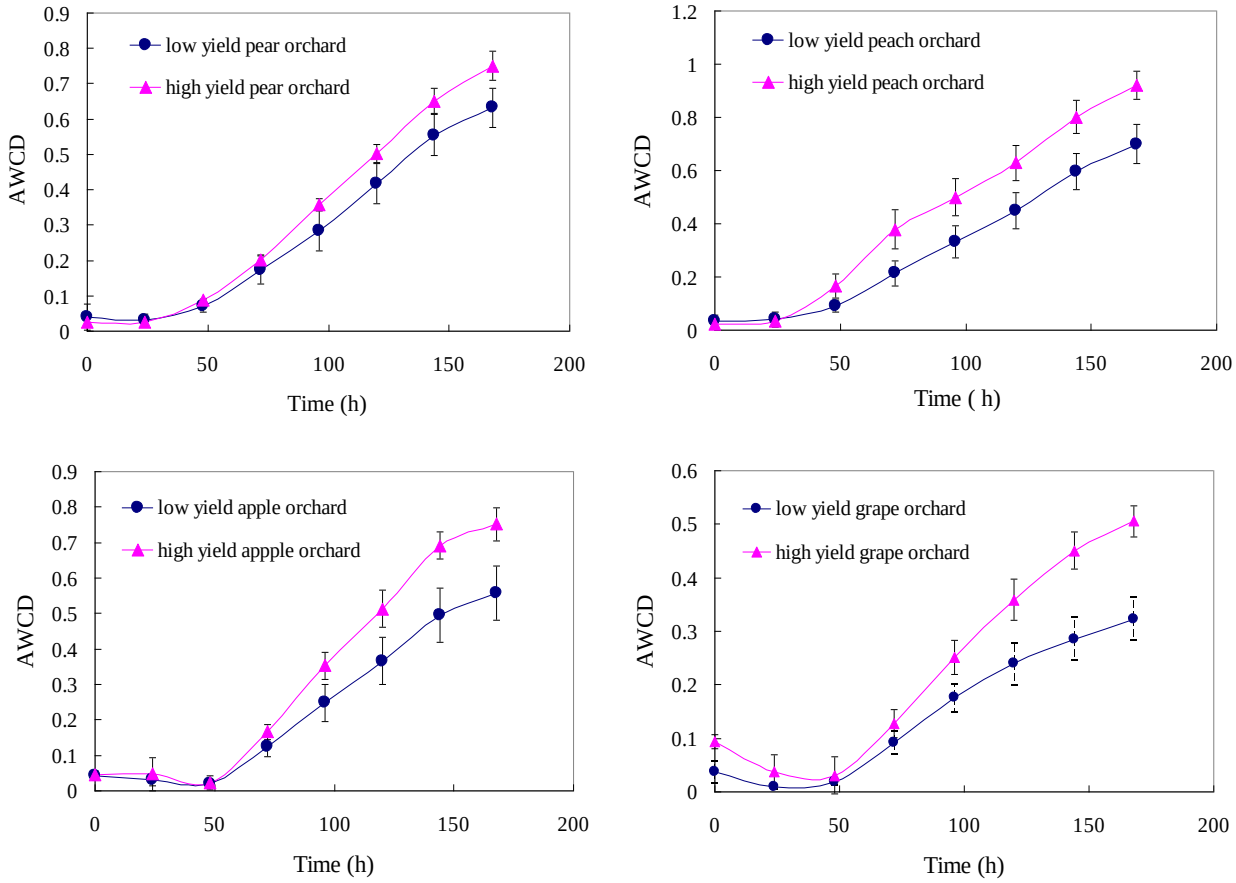

Figure 1. Substrate utilisation patterns on soils from four different fruit trees between high-yield and low-yield orchard of soil microbial communities as indicated by AWCD at 590 nm.

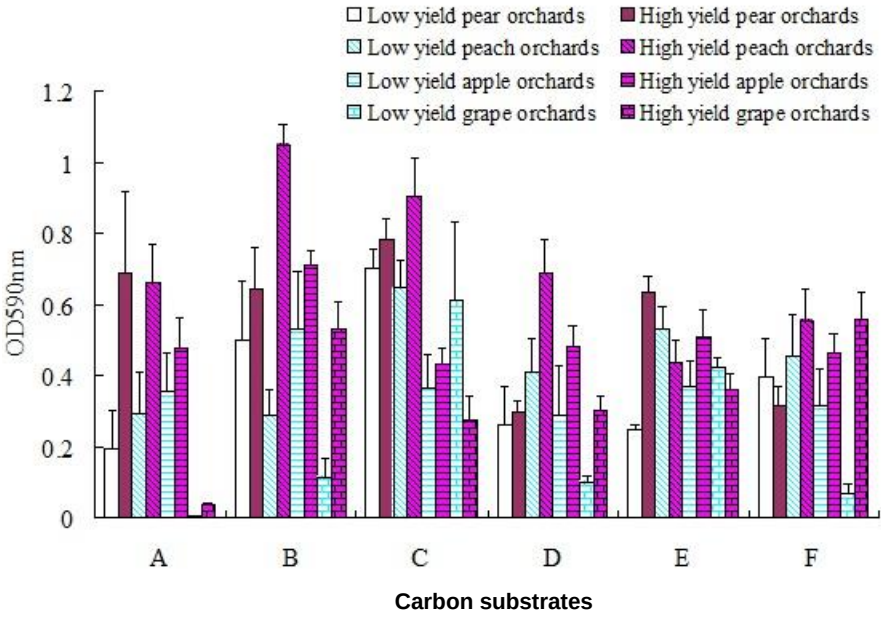

Figure 2. Average utilisations of different carbon substrates by soil microorganisms in the high-yield and low-yield orchards in four kinds of fruit orchards based on 120 h incubations. A–F denotes amines/amides, amino acids, carbohydrates, carboxylic acids, miscellaneous and polymers, respectively.

Table 2. Diversity patterns for soils of high-yield and low-yield orchards.

Soil type	Shannon–Wiener	Evenness	Simpson's diversity
High-yield apple	2.72 ± 0.09	0.85 ± 0.03	0.92 ± 0.01
Low-yield apple	2.27 ± 0.63	0.81 ± 0.07	0.84 ± 0.12
High-yield grape	2.53 ± 0.12	0.80 ± 0.01	0.88 ± 0.01
Low-yield grape	2.12 ± 0.38	0.78 ± 0.06	0.81 ± 0.05
High-yield pear	2.33 ± 0.21	0.74 ± 0.04	0.86 ± 0.02
Low-yield pear	2.34 ± 0.43	0.77 ± 0.08	0.85 ± 0.07
High-yield peach	2.79 ± 0.25	0.84 ± 0.06	0.92 ± 0.01
Low-yield peach	2.64 ± 0.20	0.84 ± 0.05	0.91 ± 0.02

Table 3. Total PLFAs, PLFAs of bacteria and fungi, PLFAs of Gram-positive bacteria (GP), PLFAs of Gram-negative bacteria (GN), ratio of GP/GN, Fungi and fungi/bacteria extracted from soil of four types of fruit orchards (expressed in nmol g^{-1} soil). The standard deviation of the mean is given in brackets.

Soil type	Bacterial	GP	GN	GP/GN	Fungi	Fungi/bacteria	Total PLFAs
High-yield peach	7.6 (0.8)	3.4 (0.1)	4.1 (1.2)	0.86 (0.2)	2.0 (0.2)	0.26 (0.0)	17.5 (2.4)
Low-yield peach	3.3 (1.3)	1.5 (0.8)	1.5 (0.4)	0.98 (0.28)	0.8 (0.3)	0.23 (0.01)	9.3 (2.1)
High-yield pear	10.9 (0.8)	3.0 (0.1)	7.5 (0.8)	0.40 (0.04)	2.5 (0.8)	0.23 (0.09)	22.7 (1.2)
Low-yield pear	13.1 (2.7)	6.1 (2.7)	6.7 (0.4)	0.91 (0.36)	1.0 (0.1)	0.08 (0.01)	18.8 (1.1)
High-yield apple	32.2 (6.9)	16.5 (2.7)	14.8 (4.3)	1.15 (0.17)	1.9 (0.7)	0.06 (0.01)	46.7 (10.2)
Low-yield apple	22.6 (5.2)	12.1 (3.2)	9.7 (2.0)	1.23 (0.16)	1.8 (0.8)	0.08 (0.02)	39.6 (9.8)
High-yield grape	23.96 (1.0)	6.7 (0.3)	15.8 (0.8)	0.42 (0.00)	4.9 (0.2)	0.20 (0.00)	47.6 (1.6)
Low-yield grape	9.0 (3.7)	3.3 (0.7)	5.1 (2.5)	0.7 (0.3)	0.9 (0.5)	0.1 (0.0)	14.8 (6.6)

plant productivity. Soil quality is based on physical–chemical properties (e.g. bulk density, temperature, pH, CEC, organic carbon, TN, humic-like substances, AP and AK) and is useful in assessment of soil fertility. Biological parameters that indicate microbial biomass and activity are also useful as indicators of soil quality. Microbial communities are critical components of soil, and microorganisms are involved in nutrient release and organic matter decomposition. It is still unclear whether microbial organisms are correlated with fruit yield. In order to address this question, soil microbial community structure and fruit yields were investigated in field fruit-production systems and compared with SCSU patterns and PLFAs profiles. Four types of high- and low-yield fruit orchard soils were tested for soil chemical and physical properties and microbial community structures. The present experiment demonstrated that the soils of organic systems in high- and low-yield orchards showed difference on microbial community and activity. On the basis of substrate utilisation patterns in soils (Figure 1), AWCD was consistently higher in soil of high-yield than low-yield orchards of the same type of fruit. AWCD indicated the overall rate of the community-level response in the Biolog plate, and infers greater metabolic functioning of the soil bacterial community in high-yield than in low-yield orchards. The diversity patterns (Shannon–Wiener and Simpson's diversity) were greater for soils of high-yield than of low-yield orchards (Table 2), indicating more abundant of microbes in soil of high-yield orchards.

The Biolog method indicates the activity of fast-growth bacteria or eutrophic bacteria only, and cannot reveal the activity of slow-growth or uncultured bacteria. Thus only a part of soil microbial characteristics were shown by this method. To fully understand soil microbial community structure, other assay methods are required. Analysis of microbial PLFAs is an effective tool for characterising microbial communities, and provides a quantitative profile of the microbial population. The PLFAs data indicate that microbial biomass was higher in high-yield compared to low-yield orchards. It has been reported that the amount of nutrients held in microbial biomass and amounts of mineralisable nutrients in the soil are positively correlated (Carter and MacLeod, 1987; Dalal and Mayer, 1987). The differences in microbial biomass may have implications for nutrient availability to crops. High microbial biomass and activity often lead to high nutrient availability to crops (Zaman et al., 1999; Tu et al., 2006). Thus we conclude that soil of high-yield orchards had higher nutrient availability than that of the low-yield orchards. The soil in relatively higher fruit-production orchards could have more microbial biomass in different type of fruit tree orchards. Higher microbial biomass was positively influenced to the fruit production, indicating that microbial biomass can be a valuable ingredient of soil.

The use of carefully identified 'paired sites' indicated some differences between paired sites for each kind of

fruit tree. Soil bulk density was consistently greater in the low-yield than the high-yield orchards. Pankhurst (1995) reported that the soils showing yield decline had a high bulk density and low microbial biomass, indicating that there may be populations of detrimental soil organisms affecting the growth and health of the plant root system. High soil bulk density indicates soil compaction and less air in the soil (important for plant roots and microorganisms). Lower pH was also recorded on low-yield orchards, except for grape. But the other soil (except soil bulk density, the pH) properties showed no clear trends between the high-yield and low-yield orchards and their corresponding fruit yields (Table 1). Therefore, we could attribute the higher yields of the organic orchards mainly to the higher abundant of soil microbial biomass, which can help to supply the nutrients for plant growth and development over time.

Since the abundant of soil microbial communities is closely related to the fruit yields in organic orchards, we can adjust the crop management to improve the soil microbial diversity and so ameliorate soil quality, such as, using organic amendments to increase the microbial biomass and microbial activity (Huang et al., 2011). We can also incorporate or subsequently manipulate these beneficial microbial populations in the field over the course of a growing season(s). Further studies will be performed for understanding of the mechanisms of soil microbial community promoting fruit yield.

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Full Length Research Paper

Gelatinase production, antimicrobial resistance and pheromone response of conjugative plasmids of *Enterococcus faecalis* isolated from Egypt

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Published data concerning correlation between virulence factors and resistance markers is relatively scarce. The aim of this study was to determine the relationship between antibiotic resistance, gelatinase production and pheromone response in the *Enterococcus faecalis* pathogens isolated from Egypt. Out of the 19 *E. faecalis* clinical isolates, 5 were able to produce gelatinase enzyme. These virulent isolates were multidrug resistant and showed high level vancomycin resistance. They were tested for mating ability. They cotransfered pheromone response genes together with vancomycin resistance determinants and gelatinase production to sensitive recipient strains. The *gel E* gene was detected in all donor isolates and their corresponding transconjugants phenotypically and genotypically. The PCR amplicons of a heavy gelatinase producing isolate and its transconjugants were subjected to sequencing. Significant homology was detected with OG1RF strain following sequence search with a GenBank database. Statistical analysis revealed significant positive direct correlation between vancomycin- and chloramphenicol-resistance, and gelatinase production in *E. faecalis* isolates.

Key words: Correlation, *Enterococcus faecalis*, gelatinase, antimicrobial resistance, conjugation.

INTRODUCTION

Enterococci have emerged as very important nosocomial pathogens, and this is attributed, among other factors, to their broad natural and acquired resistance to antimicrobial agents, including glycopeptides, vancomycin and teicoplanin (Cetinkaya et al., 2000; Gold, 2001;Oskoui and Farrokh, 2010). Although a lot of *Enterococcus* species have been identified, only two are responsible for the majority of human infections, that is, *Enterococcus faecalis* and *Enterococcus faecium*. The most common nosocomial infections produced by these organisms are urinary tract infections (associated with instrumentation and antimicrobial resistance), followed by intra-abdominal and pelvic infections. They also cause surgical wound infections, bacteraemia, endocarditis, neonatal sepsis and rarely meningitis. A major reason why these organisms sms survive in hospital environment is the intrinsic resistance to several commonly used antibiotics and, perhaps more importantly, their ability to acquire resistance to all currently available antibiotics, either by mutation or by receipt of foreign genetic material through the transfer of plasmids and transposons. The emergence of vancomycin-resistant enterococci (VRE) is a cause of concern, as once established, it is very difficult to control (Sood et al., 2008). There is initial, usually asymptomatic colonization of gastrointestinal tract by enterococcal strains possessing various traits, such as antibiotic resistances, cytolytic toxin genes, or possibly aggregation substance or the protease gelatinase upon hospital admission. (Morris et al., 1995). A report by Kuhnen et al. (1988) showed that the most frequent isolates from two intensive

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care units were *Enterococcus* (formerly Streptococcus) *faecalis* subsp. liquefaciens (the subspecies named liquefaciens are, by definition, gelatinase producers).

This article highlights gelatinase production by enterococci, along with their antimicrobial resistance. We focused on the probability of cotransfer of pheromone responses as well as some antimicrobials from resistant gelatinase producing *E. faecalis* isolates to plasmidless strains.

MATERIALS AND METHODS

Test organisms

A total of 19 *E. faecalis* isolates were recovered from 23 stool specimens, collected from the clinical pathology laboratory of Tanta University Hospitals during summer season. Purity and identity of the clinical isolates were confirmed (Koneman et al., 1992; WHO, 2003). An isolate of *Escherichia coli* L99 used as a plasmid molecular weight marker was obtained from Department of Microbiology, Faculty of Pharmacy, Tanta University. *Staphylococcus aureus* (RN4220) and *E. faecalis* (JH2-2) were used as recipient strains in this study. Both recipients were obtained from Department of Clinical Analysis and Toxicology, Faculty of Pharmaceutical Science, University of Sao Paulo, Brazil.

Chemicals and culture media

All chemicals used were purchased from Sigma, USA while culture media were purchased from Oxoid, UK. Dream green PCR master mix, 2X, (Fermentas, USA), DNA ladder (100 pb) and nuclease free water (Fermentas, USA) were used. Forward and reverse primers, used for amplification, were synthesized by a custom primer service (Fermentas, USA). All primers were reconstituted with nuclease free water to obtain a final concentration of 100 picomle/µl (stock solution).

Detection of gelatinase producing *E. faecalis* isolates

Phenotypic detection

Production of gelatinase was determined on nutrient agar containning 3% gelatin. Single colonies were streaked onto plates, grown overnight at 37°C, then 1% tannic acid was poured on the surface of the medium to precipitate the unhydrolyzed gelatin, leaving a transparent halo around the colonies indicating positive result (Eaton and Gasson, 2001; Lopes et al., 2006).

Genotypic detection of the virulence plasmid

PCR detection of *gel E* gene located on the virulence plasmid in the tested isolates was carried out according to Creti et al. (2004). The primers that were used to check for the presence of this gene were: *gel E* genes, F- 5'-ACCCCGTATCATTGGTTT-3'-, 5'-ACGCATTGCTTTTCCATC-3'. The strain used as a negative control was: *E. faecalis* (JH2-2). When the green master mix was brought to a final volume of 25 µl, each reaction contained 0.05 units/µl of Taq DNA polymerase, PCR buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP and the plasmid DNA were used. All PCR amplifications were performed in a TC-3000G Thermocycler (TECHNE, UK) using the following PCR programme: 5 min at 95°C; 30 cycles of 60 s at 95°C, 60 s at 52°C, 60 s at 72°C and 10 min at 72°C. The PCR products were run on a 0.9% A 9539 agarose gel (Sigma, USA). The amplicon size was 405 bp (Creti et al., 2004).

Susceptibility of the virulent isolates to different antimicrobial agents

Susceptibility of gelatinase producing *E. faecalis* isolates to different antimicrobials was performed using break point concentration of agar diffusion technique according to the procedure described by the Clinical and Laboratory Standard Institute guidelines (CLSI, 2010).

Plasmid DNA study

Plasmid DNA analysis

Plasmids of selected *E. faecalis* isolates were extracted by modified alkaline lysis method (Alebouyeh et al., 2005) and they were purified using the QIAprep Spin Miniprep kit (Qiagen). Profiles were compared after electrophoresis on 1% agarose gel. The plasmid sizes were measured by comparing their mobility with those of plasmids of known size, like the ones from *E. coli* (L99).

Mating study

A plasmid-free, esculin fermentor, rifampicin- and fusidic acid-resistant (F^-, Esc^+, RIF^r, FUS^r) strain of *E. faecalis* (JH2-2) was used as a recipient strain. *S. aureus* (RN4220) was also used as another recipient strain in this study and it is a plasmid-free, mannitol fermentor, chloramphenicol-resistant (F^-, Man^+, CHL^r) strain. Filter mating was performed using a 1:1 donor-recipient mixture. Briefly, 0.5 ml of each overnight culture of the gelatinase producing isolates (donors) and one of the recipients in brain heart infusion (BHI) broth were mixed and harvested using 0.45 µm-pore-size filters and incubated on BHI agar plates at at37°C for 24 h. Cells were harvested and diluted in BHI broth and grown for 2 days at 37°C on BHI agar plates. For selection of transconjugants, enterococcal transconjugants were selected on bile esculin azide agar supplemented with one of the antimicrobials to which the donor strain is resistant and 100 µg/ml of rifampicin and 25 µg/ml of fusidic acid (to which the JH2-2 strain is resistant). Staphylococcal transconjugants were selected on mannitol salt agar supplemented with one of the antimicrobials to which the donor strain is resistant and 100 µg/ml of chloramphenicol (to which the RN4220 strain is resistant). The frequency of transfer was expressed relative to the number of donor cells (Davis et al., 1980; Lyon et al*.,* 1983; Birnboim and Doly, 1979).

Detection of virulence factor and antimicrobial resistance markers in transconjugants

The transconjugant colonies were picked with tooth pick and cultured onto the selective media containing break points of each of the tested antimicrobials and incubated overnight. Transconjugants were also tested for gelatinase production as described before. Plasmid analysis of the *S. aureus* as well as *E. faecalis* transconjugants was carried out as described earlier. Gelatinase producing donors and their transconjugants were all tested for their ability to aggregate when exposed to a cell free culture filtrate of JH2-2. This method tests for any pheromone response as described by Ike and Clewell (1984).

Curing of plasmids

Traits to eliminate plasmids of the transconjugants were done using ethidium bromide (E Br) at elevated temperature 42°C. Two fold serial dilution of E Br were prepared in Luria Bertani (LB) broth. Aliquots of 100 µl of 10^7 CFU/ml suspension of the test transconjugant were inoculated into the serial E Br dilutions. The tubes were gently shacked and incubated at 42°C for 24 h. After overnight incubation, the tubes were inspected for the presence of turbidity.

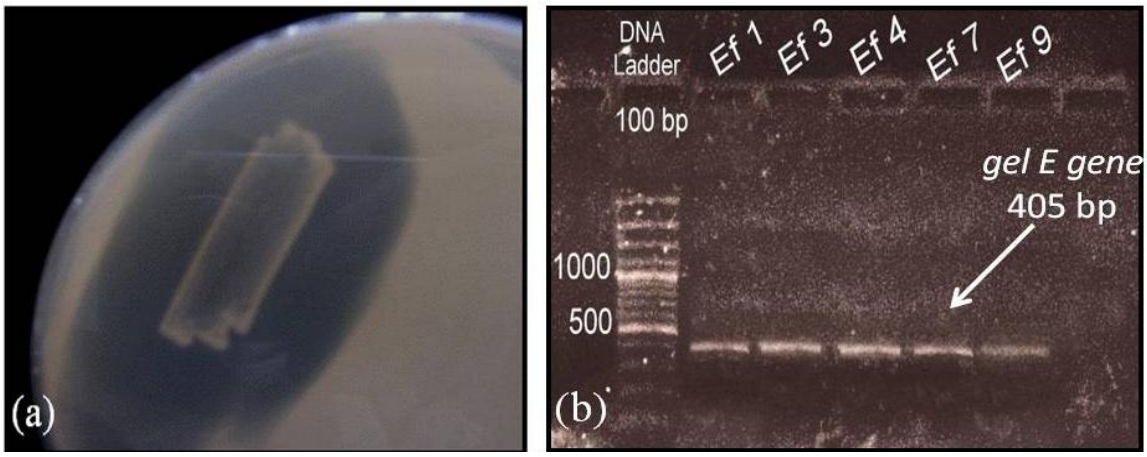

Figure 1. Detection of gelatinase production by *E. faecalis* isolates, (a) gelatin agar showing clear halo zone around the colonies, (b) electrophoregrams of the amplified PCR products of *gel E* gene.

The subinhibitory concentration of E Br was taken as the highest concentration showing turbidity. Hundred microliter from subinhibitory concentration for each isolate were subcultured by aseptically spreading on the surface of LB agar plates. After overnight incubition at 37°C, each of the resultant colonies was picked with tooth pick and subcultured on LB agar plates containing the break point concentration of the tested antimicrobials. Control plate containing LB agar without antimicrobial agent was simultaneously subcultured. Colonies which grew on the control plates but failed to grow on antimicrobial agent containing plate were detected as the cured cells. Detection of the virulence factors was carried out on the cured derivatives as previously mentioned. The plasmids of each cured strain were prepared, electrophoresed and photographed as mentioned before (Darfeuille-Michaud et al., 1992; Mandal et al., 2003).

Nucleotide sequencing and analysis

Nucleotide sequence data was obtained by stepwise sequencing. Initially, forward primers of *gel E* gene were used for sequencing. As new sequence data became available, customized synthetic primers sequencing reaction products were analyzed with the Applied Biosystems model 373A DNA sequencer (Applied Biosystems, Inc.). Sequencing was performed at laboratories of the City of Scientific Research and Technological Science at New Borg ELarab, 21934 Alexandria, Egypt; using the Big dye terminator v.3.0 sequencing kit and a 3700 DNA analyzer (Applied Biosystem, Foster City, CA, USA). Nucleotide sequence analysis & searches for homologous DNA sequences in the GeneBank database libraries were performed with the program BLASTN 2.2.26+ as well as BLASTX 2.2.26+ (http://www.ncbi.nlm.nih.gov/BLAST/Blast). The GeneBank accession number CP002621.1 was assigned to the nucleotide sequences of *gel E* gene of the selected donor and their transconjugants. G blocks were determined for phylogenetic analysis http://www.phylogeny.fr/version2_cgi/simple id=653280cc12fd1fb407ea532380ae4864&tab_index=6.

Statistical analysis

The data was analyzed with SPSS version 15 statistical software package SPSS (SPSS, Inc., Chicago, IL). Correlations between virulence factor and resistance markers were evaluated using the Pearson's *r* coefficient.

RESULTS

Detection of gelatinase production by *E. faecalis* isolates

Five out of 19 (26.3%) *E. faecalis* isolates were gelatinase producers. Production of gelatinase was detected as a clear zone around the growth of the test isolates as shown in Figure 1a. PCR experiment was carried out on the plasmid DNA of gelatinase producing *E. faecalis* isolates. Bands with molecular weight 405 bp were detected in the electrophoregram shown in Figure 1b, indicating the presence of *gel E* gene.

Susceptibility of the virulent clinical isolates to different antimicrobial agents

As shown in Table 1, all gelatinase producing *E. faecalis* isolates were resistant to SSS and VAN. Moreover, all the 5 isolates showed vancomycin resistance (VAN A type) according to the MIC results (data not shown). On the other hand, AMP, IMP, STR and TET were active on all the tested isolates as shown in Table 1.

Analysis of plasmid profiles of gelatinase producing *E. faecalis* isolates

Plasmid profiles and resistance patterns of selected isolates

The plasmid profiles of the 5 gelatinase-producing *E. faecalis* isolates are presented in the electrophoregram as shown in Figure 2. Four plasmid sizes were detected among the tested *E. faecalis* isolates including 140, 106, 1.6 and 1.2 MDa. The high molecular weight plasmids 140 and 106 MDa were present in all the studied isolates.

Table 1. Incidences of antimicrobials resistance among gelatinase producing *E. faecalis* isolates.

AMA* break point** (µg/ml)	*E. faecalis* (n = 5)
AMP ≥ 32	0
IMP ≥16	0
RAD ≥64	4
CTX ≥64	2
FEP ≥32	2
STR ≥64	0
TET ≥16	0
CHL ≥32	4
RIF ≥4	4
ERY ≥ 8	2
AZ ≥ 2	2
SSS ≥512	5
TRI ≥ 16	2
SXT ≥ 80	2
VAN ≥ 32	5
NOR ≥ 16	2
CIP ≥ 4	2
ENX ≥ 4	3
MOX ≥8	2

*AMA: Antimicrobial agents, AMP: Ampicillin, IMP: Imipenem, RAD: Cephradine, CTX: Cefotaxime, FEP: Cefepime, STR: streptomycin, TET: Tetracycline, CHL: Chloramphenicol, RIF: Rifampicin, ERY: Erythromycin, AZ: Azithromycin, SSS: Sulfamethoxazole, TRI: Trimethoprime, SXT: Sulfamethoxazole /Trimethoprime, VAN: Vancomycin, NOR: Norfloxacin, CIP: Ciprofloxacin, ENX: Enoxacin, MOX: Moxifloxacin.
**Break points were done according to CLSI, 2010.

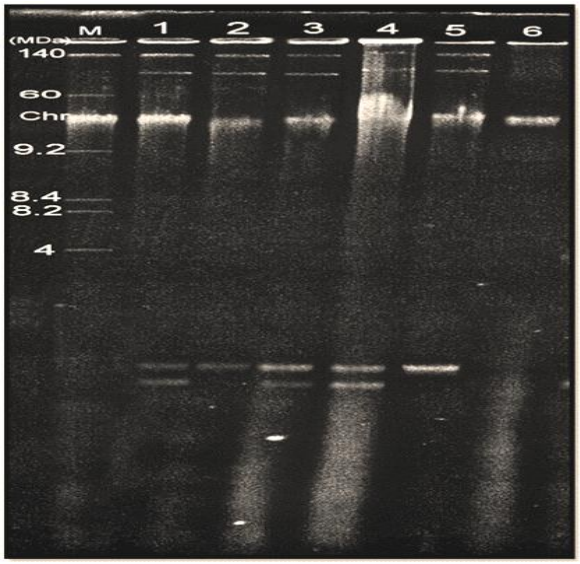

Figure 2. Electrophoregrams of virulent *E. faecalis* isolates. Lanes 1-5: gelatinase producing isolates (Ef 3, 1, 4, 7 and 9, respectively); Lane 6: JH2-2, a negative control of *E. faecalis* strain; Lane M: molecular weight marker.

Conjugal transfer of the plasmids of gelatinase producing isolates

Three multiresistant gelatinase producing *E. faecalis* isolates were selected, one from each resistance pattern, and subjected to conjugation and curing experiments. The plasmid profiles of the donors, transconjugants and the cured derivatives are presented in the electrophoregrams shown in Figure 3.

As shown in Table 2, all the donors plasmids were transferable except for 140 MDa. It was noted that 106 MDa plasmid was the most common one transferred to all transconjugants. Conjugation failed when dEf 9 was incubated with *S. aureus* RN4220 recipient strain (Table 2).

As recorded in Table 2, the resultant transconjugants acquired some resistance markers from the donors including RAD, VAN, CHL, TRI or SSS. All these resistance markers were lost after curing except for cEf 7. The data shown in Table 2 revealed also that gelatinase production, which is a virulence factor presented by the tested donors of *E. faecalis* isolates, was transferred to the corresponding transconjugants. All gelatinase-producing donors and the corresponding transconjugants exhibited a clumping response upon exposure to a cell free solution of pheromones comprising an *E. faecalis* JH2-2 culture filtrate.

Detection of gelatinase transfer in dEf 3 and tEf 3

Being heavy gelatinase producers, dEf 3 isolate and its transconjugants, tEf 3 and tS 3, were selected for the PCR experiment and sequence analysis.

PCR amplification of gel E gene

All of the dEf3, tEf3 and tS3 were selected and subjected to the PCR experiment. Positive amplicons of *gel E* gene were detected at 405 bp as shown in Figure 4. The resultant amplicons were subjected to sequencing.

Analysis of the obtained sequence

Single sequence analysis: Analysis of nucleotide sequences of dEf 3, tEf 3 and tS3 amplicons revealed that these sequences belong to *E. faecalis* (OG1RF strain). Moreover, the OG1RF strain is gelatinase producer (Gulhan et al., 2006). The percentage of identity of the donor sequence to that of OG1RF strain was 86%, while those of the transconjugants (tEf 3 and tS3) were 90 and 89%, respectively. In addition, significant E- values were recorded for the donor ($5e^{-69}$) and also for the trans-conjugants ($2e^{-108}$ & $3e^{-99}$), respectively, as recorded in Tables 3, 4 and 5. The program BLASTX 2.2.26+ was used to translate

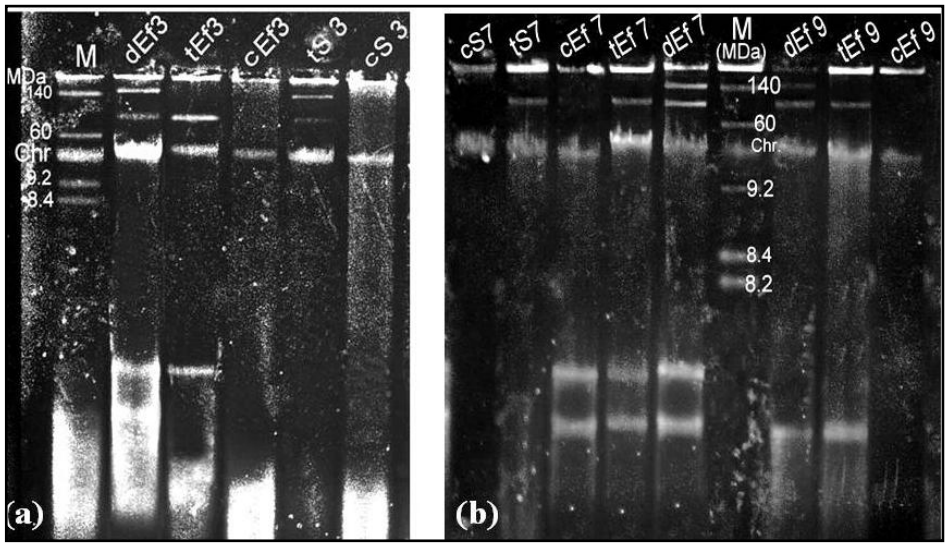

Figure 3. Electrophoregrams showing the plasmid profiles of the donors, transconjugants and selected cured derivatives of (a) Ef 3 isolate, (b) Ef 9 and dEf 7 isolates of gelatinase producing *E. faecalis*. Lane M; molecular weight marker.

Table 2. Gelatinase production, pheromone response, plasmid profiles and resistance patterns of donors, transconjugants and cured derivatives of selected *E. faecalis* isolates.

Isolate* code	Gelatinase production	Pheromone response	Plasmid** profiles	Resistance patterns
dEf 9	+	+	140- **106**- 1.2	ENX- SSS-VAN
tEf 9	+	+	**106** – 1.2	VAN
cEf 9	-		--	--
dEf 7	+	+	140- **106**- 1.6- 1.2	RAD-VAN-CHL-TRI-SSS-SXT
tEf 7	+	+	**106**- 1.6- 1.2	RAD-VAN-CHL-TRI-SSS
cEf 7	-	-	1.6- 1.2	TRI-SSS
tS 7	+	+	**106**	VAN-CHL
cS 7	-	-	--	--
dEf 3	+	+	140- **106**- 1.6	RAD-CTX-FEP-VAN-NOR-CIP-ENX-MOX-CHL-ERY-AZ-SSS
tEf 3	+	+	**106**- 1.6	VAN -CHL-SSS
cEf 3	-	-	--	--
tS 3	+	+	**106**	VAN-CHL
cS 3	-	-	--	--

*dEf: Donor of *E. faecalis*; tEf:transconjugant of *E. faecalis*; cEf: cured *E. faecalis*; tS: transconjugant of *S. aureus*; cS: cured *S. aureus.* ***Bold** number refer to the common plasmid transfered.

the nucleotide sequence of the donor to protein and to detect its function. It was found that the sequence belong to gelatinase enzyme, gluzincin superfamily and peptidase M-4. Putative. It was found that the sequence belong to gelatinase enzyme, gluzincin superfamily and peptidase M-4. Putative conserved domains of this enzyme are identified within the input sequence starting from nucleotide 217 to nucleotide 360 for the donor amplicon. A representative graphic showing the position of each conserved domain of the donor isolate was shown in Figure 5.

Multiple sequence analysis:

DNA alignments were carried out according to the phylogenitic software of MUSCLE (G blocks). Figure 6 shows an alignment between the homologous sequences of

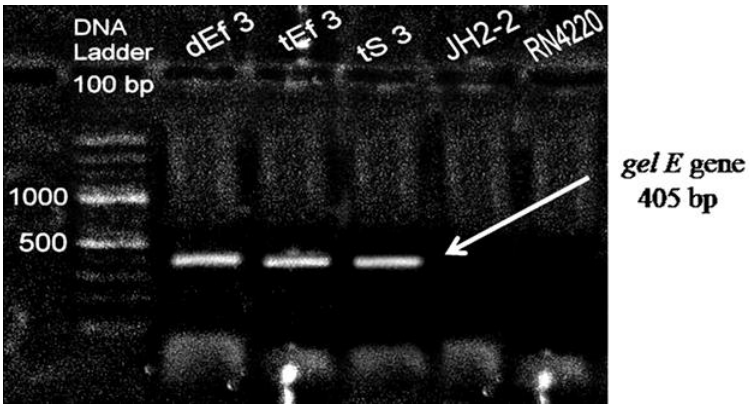

Figure 4. Electrophoregrams of *gel E*- amplicons of donor (dEf 3) of *E. faecalis* isolates, and its transconjugants (tEf 3 and tS3). Lane JH2-2 and RN4220: negative controls.

Table 3. A hit list from a BLAST search with gelatinase producing *E. faecalis* (dEf 3) as the query against the nr database. Black arrow points to the most significant subject in the database.

Accession	Description	Maximum score	Total score	Query coverage (%)	E-value	Maximum identity (%)
CP002621.1	*Enterococcus faecalis* OG1RF, complete genome	270	270	56	5e-69	86
M37185.1	*Enterococcus faecalis* gelatinase (*gelE*) gene, complete cds	270	270	56	5e-69	86
JN246675.1	*Enterococcus faecalis* strain LN68 Fsr and gelE-sprE operons, complete sequence	265	265	56	3e-67	85
HE574483.1	*Enterococcus faecalis sprE, gelE, fsrC, fsrB, fsrD* and fsrA genes, strain LN68	265	265	56	3e-67	85
CP002491.1	*Enterococcus faecalis* 62, complete genome	265	265	56	3e-67	85
EF105504.1	*Enterococcus faecalis* GM gelatinase (*gelE*) gene, complete cds	265	265	56	3e-67	85
D85393.1	*Enterococcus faecalis* gelatinase (*gelE*) DNA, complete cds	265	265	56	3e-67	85
EU862241.3	*Enterococcus faecalis* strain H81 gelatinase (*gelE*) gene, partial cds	259	259	56	1e-65	85
AE016830.1	*Enterococcus faecalis* V583, complete genome	254	254	56	5e-64	85
FP929058.1	Enterococcus sp. 7L76 draft genome	243	243	56	1e-60	84
FJ858146.1	*Enterococcus faecium* strain QSE32 fsr operon, complete sequence; and GelE (*gelE*) and SprE (*sprE*) genes, complete cds	243	243	56	1e-60	84

Table 4. A hit list from a BLAST search with gelatinase producing *E. faecalis* (tEf 3) as the query against the nr database. Black arrow points to the most significant subject in the database.

Accession	Description	Maximum score	Total score	Query coverage (%)	E-value	Maximum identity (%)
CP002621.1	*Enterococcus faecalis* OG1RF, complete genome	401	401	82	2e-108	90
M37185.1	*Enterococcus faecalis* gelatinase (*gelE*) gene, complete cds	401	401	82	2e-108	90
JN246675.1	*Enterococcus faecalis* strain LN68 Fsr and gelE-sprE operons, complete sequence	396	396	82	7e-107	89

Table 4. Contd.

HE574483.1	*Enterococcus faecalis sprE, gelE, fsrC, fsrB, fsrD* and *fsrA* genes, strain LN68	396	396	82	7e-107	89
EF105504.1	*Enterococcus faecalis* GM gelatinase (*gelE*) gene, complete cds	396	396	82	7e-107	89
D85393.1	*Enterococcus faecalis* gelatinase (*gelE*) DNA, complete cds	396	396	82	7e-107	89
CP002491.1	*Enterococcus faecalis* 62, complete genome	390	390	82	3e-105	89
EU862241.3	*Enterococcus faecalis* strain H81 gelatinase (*gelE*) gene, partial cds	379	379	82	7e-102	88
AE016830.1	*Enterococcus faecalis* V583, complete genome	379	379	82	7e-102	88
FP929058.1	Enterococcus sp. 7L76 draft genome	374	374	82	3e-100	88
FJ858146.1	*Enterococcus faecium* strain QSE32 fsr operon, complete sequence; and GelE (*gelE*) and SprE (*sprE*) genes, complete cds	374	374	82	3e-100	88

Table 5. A hit list from a BLAST search with gelatinase producing *S. aureus* (tS3) as the query against the nr database. Black arrow points to the most significant subject in the database.

Accession	Description	Max score	Total score	Query coverage (%)	E-value	Maximum identity (%)
→ CP002621.1	*Enterococcus faecalis* OG1RF, complete genome	370	370	99	3e-99	89
M37185.1	*Enterococcus faecalis* gelatinase (*gelE*) gene, complete cds	370	370	99	3e-99	89
JN246675.1	*Enterococcus faecalis* strain LN68 Fsr and gelE-sprE operons, complete sequence	364	364	99	2e-97	89
HE574483.1	*Enterococcus faecalis sprE, gelE, fsrC, fsrB, fsrD* and *fsrA* genes, strain LN68	364	364	99	2e-97	89
EF105504.1	*Enterococcus faecalis* GM gelatinase (*gelE*) gene, complete cds	364	364	99	2e-97	89

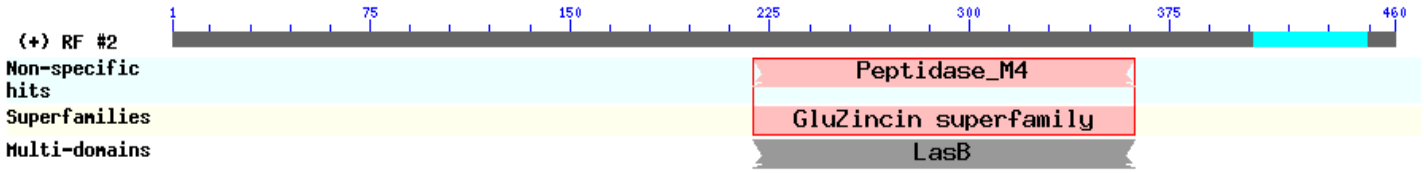

Figure 5. Graphical summary for conserved domains of seq. 11 (dEf 3).

nucleotides from gelatinase producing *E. faecalis* donor (dEf 3), tEf 3 and tS 3. The percentage of identity of the 3 sequences was 66%. Six selected G blocks were determined. Flank positions of these blocks are [63 75] [77 172] [186 252] [254 289] [293 374] [377 388].

Phylogenetic tree:

To investigate the genetic relationships between the donor *E. faecalis* dEf 3 (seq. 11), the *E. faecalis* transconjugants tEf 3 (seq. 12) and the *S. aureus* transconjugants tS 3 (seq. 15), an amplified plasmid DNA of *gel E* gene was used. The phylogenetic tree derived from these data (Figure 7) shows that the transconjugants belong to genetic group different from that of the donor. Moreover, it was noted that both transconjugants belong to the same genetic group (carry the same genetic characteristics). In addition, *E. faecalis* transconjugants (seq. 12) is closer to the donor (seq. 11), than *S. aureus* transconjugants (seq. 15) as shown Figure 7.

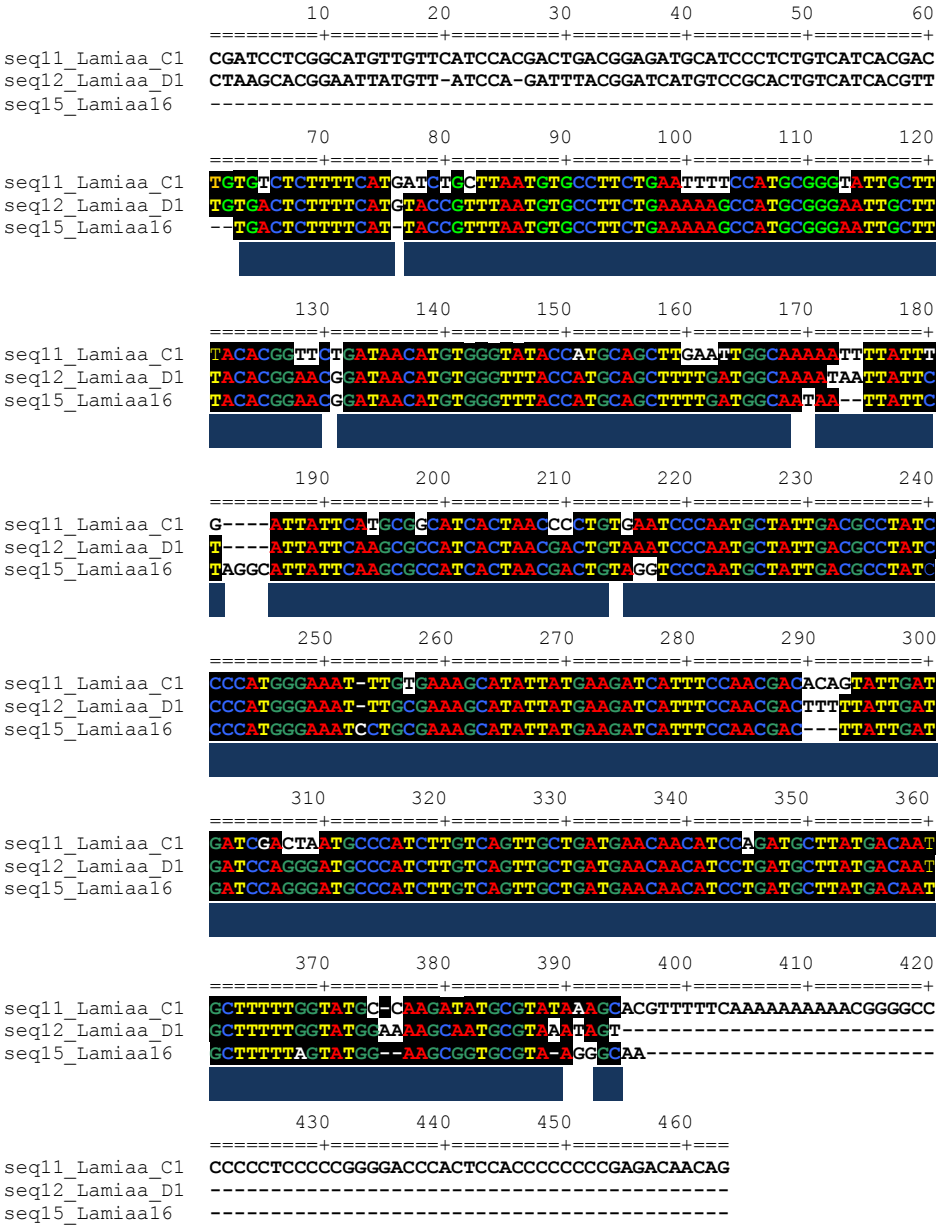

Figure 6. Multiple sequence alignment using MUSCLE software of the donor (dEf3), enterococcus transconjugant (tEf3), and staphylococcus transconjugant (tS3). Appropriate use of color can highlight positions that are either identical in all the aligned sequences or share common physicochemical properties.

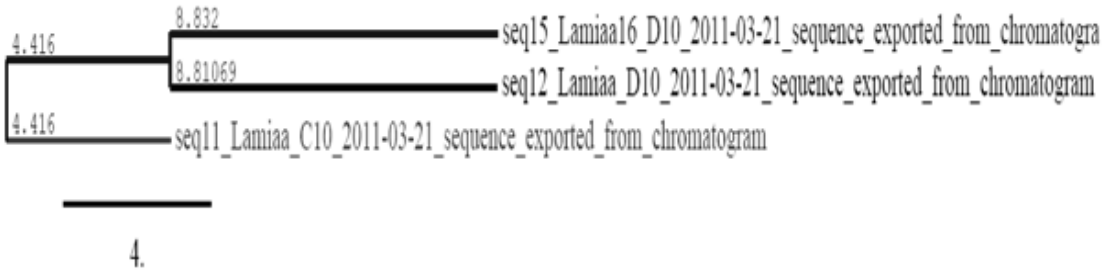

Figure 7. Phylogenetic tree of dEf3, tEf3 and tS3 showing the branch length. It is based on genes that do not match organismal phylogeny, suggesting horizontal gene transfer has occurred.

Table 6. Pearson correlation coefficient (*r*-values) between virulence factors and resistance markers.

Resistant marker	*E. faecalis* (*gel E*)
RAD	0.389
CTX	0.00
FEP	0.00
STR	0.00
TET	0.00
CHL	0.601*****
ERY	0.00
AZ	0.00
SSS	-0.433
TRI	-0.321
SXT	-0.121
VAN	0.673******
NOR	0.00
CIP	0.00
ENX	0.00
MOX	0.00

* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Correlation between gelatinase production, pheromone responses, resistance markers and plasmid profiles of the tested *E. faecalis* isolates

The inability of the 140 MDa plasmid to transfer by conjugation to the corresponding transconjugant tEf 9 was accompanied by loss of resistance to ENX and SSS. On the other hand, conjugation and curing experiments revealed that loss of 106 and 1.2 MDa plasmids from cEf 9 was accompanied by loss of resistance to VAN. Moreover, cEf 9 became unable to produce gelatinase enzyme and lost its ability to respond to pheromone. It was found that dEf 7 isolate carried 4 plasmids with molecular sizes 140 MDa, 106 MDa, 1.6 MDa and 1.2 MDa. Conjugation experiment revealed that that *E. faecalis* transconjugant tEf 7 gained106 MDa, 1.6 MDa and 1.2 MDa plasmids and became resistant to RAD, VAN, CHL, TRI and SSS while *S. aureus* transconjugant (tS 7) gained only 106 MDa plasmid that conferred resistance to VAN and CHL. The inability of the 140 MDa plasmid of the isolate dEf3 to transfer by conjugation was accompanied with the loss RAD, CTX, FEP, NOR, CIP, ENX, ERY, and AZ of resistance makers in the corresponding transconjugant while the transfer of 106 MDa and1.6 MDa plasmids was accompanied with gaining resistance to VAN, CHL and SSS, the production of gelatinase enzyme and response to pheromone by tEf 3. When the conjugation and curing experiments were done using RN4220 recipient strain, it was found that tS 3 gained 106 MDa plasmid and also it became resistant to VAN and CHL. In addition, the corresponding transconjugant produced gelatinase enzyme and exhibited a clumping response upon exposure to a cell free solution of pheromones comprising to an *E. faecalis* JH2-2 culture filtrate.

Statistical analysis of the correlation between gelatinase production and resistance markers of the *E. faecalis* isolates

Table 6 shows Pearson correlation coefficient between gelatinase production and resistance markers. Gelatinase production by *E. faecalis* isolates showed significant positive direct correlation with CHL and VAN.

DISCUSSION

The pheromone system plays an important role in the horizontal spread of genes between strains of *E. faecalis*, including those genes encoding antibiotic resistance and virulence traits (Woodford et al., 1993; Wirth, 1994). Several virulence factors, such as gelE, enterococcal surface protein (Esp), aggregation substance (AS), cytolysin, lipase and haemagglutinin are possibly asso-ciated with the colonisation and pathogenesis of enterococci. GelE is a protease produced by *E. faecalis* that is capable of hydrolysing gelatine, collagen, casein, haemoglobin and other peptides. It might play an important role in the severity of systemic diseases (Gulhan et al., 2006). In the present study, 19 *E. faecalis* isolates were recovered from stool samples. Five out of 19 (26.7%) were identified as gelatinase producers using phenotypic and genotypic techniques. Gulhan et al. (2006) reported that 68% of blood culture isolates, and 27% of community-acquired faecal isolates were gelE-positive. Also, Strzelecki et al. (2011) reported that 140 *E. faecalis* isolates (91% of the group) harbored the *gelE* gene, but only 81 isolates (53%) produced active gelatinase.

Palazzo et al. (2005) reported that fifty-one vancomycin-resistant enterococci samples isolated from different geographic regions in Brazil harbored the *vanA* gene as demonstrated by PCR analysis, and in a majority of strains, the gene was associated with a transferable plasmid of 70 kb (106.4 MDa). In the present study, 4 plasmid sizes were detected among the tested *E. faecalis* isolates including 140, 106, 1.6 and 1.2 MDa. The high molecular weight plasmids 140 and 106 MDa were present in all the studied isolates.

The glycopeptide vancomycin is the “first choice” alternative to penicillin-aminoglycoside combination for treatment of systemic enterococcal infections. The frequency of vancomycin resistant enterococcus (VRE) isolates had increased worldwide. The frequency of VRE isolates among nosocomial infections in USA was only 1% in 1989, but increased to 7.9% by 1993 (Center for Disease Control and Prevention, 1995). High (16.8%) detected rate of enterococcal urinary tract infections was reported in Canada (Low et al., 2001). Moreover, Al-Jarousha et al. (2008) reported that 66.6% of the enterococcus isolates detected in Gaza were vancomycin resis-

tant. In our study, 5 out of 19 (26.3%) *E. faecalis* isolates were vancomycin resistant and also gelatinase producers. High level vancomycin resistance (VanA) was also detected as determined by MIC test (data not shown). Similar finding was detected by Courvalin (2005), Henrique et al. (2008) and Oskoui and Farrokh (2010) who reported that VanA and VanB are widespread globally and confer the most prevalent glycopeptide resistance phenotype.

In the present study, while the 5 gelatinase producing *E. faecalis* isolates were multidrug resistant, 4 out of them were sensitive to AMP. These results were in agreement with the study of Radu et al. (2001) who reported that all the tested *E. faecalis* and *E. faecium* isolates were multidrug resistant. They showed resistance to kanamycin, nalidixic acid, VAN, CTX, ERY, CHL and STR although sensitive to AMP which was the most active agent against these isolates. This finding is of great importance since these 2 species have been associated with human infection and that ampicillin is therefore the drug of choice in the treatment of enterococcal infections. Although, penicillinase production is common in *S. aureus*, it is very rare in enterococci. Enterococci are intrinsically resistant to cephalosporins because of the presence of penicillin binding proteins (PBP) with low affinities for these agents (Woodford, 2005). The linkage between a β-lactam resistant PBP and vancomycin resistance does not appear to have occurred yet in *E. faecalis*, which may account for the sporadic detection of vancomycin resistant *E. faecalis* (Sood et al., 2008).

Enterococci possess a variety of mechanisms for transferring antibiotic resistance determinants to susceptible recipients. The pheromone-mediated conjugation systems of several plasmids have been studied including those of pDA1, pCF10, pPD1 and pAM373 (Wirth, 1994; Heaton and Handwerger, 1995; Ike and Clewell, 1984; Fujimoto et al., 1995). In the present study, tS3 and tS7 acquired a single pheromone responsive plasmid (106 MDa) together with VAN A and CHL resistance which indicates that the pheromone responsive plasmids in our hospitals frequently carried antimicrobial resistance determinants. Several other pheromone responsive plasmids have been reported previously (Wirth, 1994; Pournaras et al., 2000).

In our study, a homologous-nucleotide search with a GenBank database revealed significant sequence identities between the gelatinase encoding gene of Ef 3, tS3 and tEf3 and that of OG1RF strain. Alignment comparisons of the gelatinase of tEf3, or Ef3 tS3 with that of OG1RF, indicate 86, 89 and 90% homologies at positions 11588137-1588397, 588143-1588442 and 1588123-1588442, respectively. These regions of high homology belong to the positions of the gelatinase enzymatic active site. It was found that the nucleotides sequences of the 3 strains belong to gelatinase enzyme, gluzincin superfamily, peptidase M-4. Putative conserved domains of this enzyme are identified within the input sequence starting from nucleotide 217 to nucleotide 360 for the donor amplicon. Mäkinen et al. (1989) Su et al. (1991) and Qin et al. (2000) reported that a homologous-protein search revealed significant amino acid sequence similarities between the gelatinase of *E. faecalis* and neutral proteases from *Bacillus thermoproteolyticus*, and elastase from *Pseudomonas aeruginosa*. The amino acid sequence matrix plots of the gelatinase versus thermolysin of *B. thermoproteolyticus* and versus elastase of *P. aeruginosa* showed that each point reflects at least 40% homology within an 8-amino-acid alignment of the corresponding proteins.

In the present study, the phylogenetic tree derived from the data showed that the transconjugants belong to genetic group different from that of the donor. Moreover, it was noted that both transconjugants belong to the same genetic group (carry the same genetic characteristics). In addition, *E. faecalis* transconjugant (seq. 12) is closer to the donor (seq. 11) than the *S. aureus* transconjugant (seq. 15). It was deduced that horizontal gene transfer from an ancestor of seq.11 to the ancestor of seq.12 and seq. 15 occurred because this would most simply explain the results. The results shown of this study are similar with those reported by Baxevanis and Ouellette (2001).

It was found that these gelatinase producing isolates and their transconjugants were positive in clumping experiments and contained pheromone responsive conjugative plasmid weighing 106 MDa suggesting that the pheromone system possibly contributed to vancomycin and/or chloramphenicol resistance in our setting. The statistical analysis revealed significant positive and direct correlation between gelatinase production and resistance to vancomycin or chloramphenicol (transfer of chloramphenicol resistance in *S. aureus* was detected by MIC but data not shown). Lata et al. (2009) reported that a significant correlation was observed in the distribution of multiple-antimicrobial resistance (erythromycin-rifampicin-gentamicin, methicillin and vancomycin-gentamicin-streptomycin; $r = 0.9747$; $p = 0.0083$) and multiple-virulence factors (gelE+ esp+; $r = 0.9747$; $p = 0.0083$; gelE+ efaA+; $r = 0.8944$; $p = 0.0417$) among different Enterococcus spp.

To our knowledge, this is the first report in Egypt on the relationship between gelatinase production, vancomycin resistance and pheromone response in *E. faecalis* isolates. In conclusion, the present study showed that enterococci served as reservoir for drug resistance genes. Therefore, the conjugal transfer of gelatinase production, pheromone response, as well as VanA type of vancomycin resistant genes from enterococci to other Gram-positive bacteria like *S. aureus* isolates pose a threat to public health. This gives rise to concern that such transfer in humans under natural conditions could be feasible.

New approaches are needed to control antimicrobial resistance with respect to the use of antibiotics. The era where acute or chronic bacterial infections were treated

with ‘‘antibiotics-only’’ appears to have come to an abrupt end. It is time to intensify attention on search for compounds that can block the expression of genes encoding virulence factors or even inhibit the conjugation process. Formulations containing antimicrobial agents combined with such conjugation inhibitory compounds would be a potential solution for the problem. Finally, data of our work strongly recommend periodic evaluation of both virulence factors as well as resistance markers in *E. faecalis* isolates otherwise spread of resistance combined with virulence genes among bacterial pathogens would leave clinicians with fewer treatment options.

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Full Length Research Paper

Antibacterial properties of *Lactobacillus plantarum* isolated from fermented mustards against *Streptococcus mutans*

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Antibacterial properties of *Lactobacillus* strains isolated from traditional fermented mustard were investigated. Among the strains tested, strain B0105 was found to produce bacteriocin-like compounds against *Streptococcus mutans* BCRC 10793. Strain B0105 was identified as *Lactobacillus plantarum* by phenotypical and physiological tests as well as16S rDNA identification. The molecular weight of bacteriocin-like compounds was 3.5 and 4.7 kDa by Tris-Tricine SDS-PAGE. In addition, this bacteriocin-like exhibited a strong antibacterial activity, heat stability (15 min at 121°C) and pH stability (pH 2.0–4.0) against *S. mutans* BCRC 10793, however, it was sensitive to proteolytic enzyme. Overall, the results obtained, demonstrate that strain B0105 is able to produce bacteriocin-like compounds inhibiting *S. mutans* BCRC 10793, making it potential candidate for antibacterial agents.

Key words: *Lactobacillus plantaru*m, bacteriocin, *Streptococcus mutans*.

INTRODUCTION

Dental caries is influenced by diverse bacterial, dietary, environmental, socioeconomic and physiological risk factors and is one of the most prevalent diseases in humans (Sánchez-Pérez et al., 2004; Marthaler, 2004). One of the properties required for a caries-preventive sweetener is that it decreases fermentation of sugar to acid by oral-microorganisms, including *Streptococcus mutans* which is a primary causative agent of human dental caries (Padilla et al., 2006). *S. mutans* present good adherent capacity owing to the production of glycocalyx and are responsible for initiating the cariogenic process (Cvitkovitch et al., 2003). To control dental caries formation, antibacterial agents that reduce the number of *S. mutans* are commonly used, for exam-ple xylitol, chlorhexidine and triclosan (Fejerskov, 2004). Using such antibacterials may induce resistance in micro-organisms (Badet et al., 2004; Diekema et al., 2004). Therefore, it is important to search for new antibacterials that may control the effect of *S. mutans* (Padilla et al., 2006).

Bacteriocins are defined as antibacterial peptide or proteins generally active against closely related species (Cleveland et al., 2001). Bacteriocins from lactic acid bacteria (LAB) have attracted more interest than those from other resources (Gao et al., 2010). Antagonistic effects produced by LAB towards other organisms may play an important role in maintaining a proper microbial

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balance in intestine tract and preserving certain foods (Millette, et al., 2007; Udhayashree et al., 2012). LAB are widely used as a starter culture and play an important role in food preservation, microbial stability and aroma compounds (Ravi et al., 2011, Abriouel et al., 2012). Many novel bacteriocin-producing LAB have been isolated from various foods and characteristics of these bacteriocins have been investigated (Gao et al., 2010). The properties of bacteriocins, that is, sensitivity to proteolytic enzymes, a narrower spectrum of antibacterial activity, and the antagonism phenomena, could make bacteriocins promising chemotherapeutic agents for the treatment of dental caries. During the last few years, a large number of new LAB bacteriocins have been identified and characterized (Cizeikiene et al., 2013). However, few studies have described bacteriocins activities against *S. mutans*.

Suan-tsai is a traditional fermented mustard which is widely consumed in Taiwan. It is made from green mustard and its production is a spontaneous fermentation process by a mixed microbial population mainly composed of LAB. Some LAB from fermented foods have been described to produced bacteriocins, e.g. *Lactobacillus pentosus* B96 (Delgado et al., 2005), *Lactobacillus plantarum* BS (Elegado et al., 2004), Lactobacillus strains (Omar et al., 2008), *L. acidophilus* La-5 (Tabasco et al., 2009), *L. plantarum* strains (Sánchez-Pérez et al., 2004) and *Lactobacillus sake* C2 (Gao et al., 2010). The information related to antibacterial activity of LAB against *S. mutans* is limited. Thus, the aims of this study were to investigate the antibacterial activity of LAB against *S. mutans* isolated from traditional fermented mustard and the characteristics of bacteriocin-like compounds were also determined.

MATERIALS AND METHODS

Bacterial strains, media and growth conditions

The samples of fermented mustard were collected from southern areas of Taiwan. The fermented liquor samples (0.1 ml with serial dilutions) were spread on the surface of MRS agar and then incubated at 30°C for 1-2 days. Colonies were randomly selected and purified by re-plating on MRS agar plates. The purified colonies were primarily identified by milk agglutination. The acid producing bacteria were then identified by Gram staining and catalase tests. Only both Gram positive and catalase negative strains were selected and stored in MRS broth with 20% glycerol (v/v) at -80°C. The *Lactococcus lactis* subsp. *lactis* (BCRC 10791), *L. plantarum* (BCRC 10069) and *S. mutans* (BCRC 10793), used in determining of antibacterial activity were obtained from Bioresource Collection and Research Center (BCRC), Hsin Chu, Taiwan.

Screening for LAB producing bacteriocin from traditional fermented mustard samples

For screening of antibacterial activity of LAB isolates, 1% (v/v) of these cultures were inoculated into 50 ml MRS broth individually and incubated at 35°C for 24 h without agitation. Bacterial cell adjusted to pH 2.0 to release bacteriocins from the cell (Yang et al., 1992) were removed by centrifugation (5000 g, 20 min, 4°C) and filtration with 0.22 µm filter, then, the supernatants were examined with the diameters of inhibition zones using the agar diffusion assay method (Cizeikiene et al., 2013). Overnight test culture of *S. mutans* BCRC 10793, was diluted in saline and was inoculated in TSA agar medium to final concentration of 10^8 CFU/mL. Briefly, 200 µl of cell free supernatant were placed into wells (10.0 mm in diameter) on TSA agar plates seeded with the above test strains. After incubation at 35°C for 24 h, the diameter of inhibitory zones was determined. The pH of MRS broth was also adjusted to the same value as control. *L. lactis* subsp. *lactis* (BCRC 10791) overnight culture (10^8 CFU/mL) adjusted pH and filtrated, was used as the positive control. As a blank control, aliquots of MRS broth, treated as filtered supernatants, were used.

The pH value of the fermented supernatants was adjusted to 4.0 (Yang et al., 1992) with NaOH to eliminate the effect of low pH value and the pH of MRS broth was also adjusted to the same value as control, respectively. After adding catalase (50 U ml^{-1}, sigma), the cell free supernatants were incubated at 37°C for 3 h to eliminate the effect of hydrogen peroxide and the same supernatants without catalase were used as controls (Gao et al., 2010). Diameters of inhibition zones were recorded using *S. mutans* as indicator. *L. lactis* subsp. *lactis* (BCRC 10791) was used as the positive control. As a blank control, aliquots of MRS broth, treated as filtered supernatants, were used.

To determine the possible protein nature of the detected antibacterial substances, after eliminating hydrogen peroxide and low pH effects, the bacterial cell-free supernatants were incubated 37°C overnight with pepsin (Sigma, St. Louis, Missouri) at a final concentration of 2 mg ml^{-1} and those without enzyme treatment were used as controls (Ennahar et al., 2000). Diameters of inhibition zones were determined. *L. lactis* subsp. *lactis* (BCRC 10791) was used as the positive control. As a blank control, aliquots of MRS broth, treated as filtered supernatants, were used.

Strain identification

A PCR assay was performed using genomic DNA from strains that showed antibacterial activity against *S. mutans*. Amplification of 16S rDNA sequences by PCR was performed using the primers 27F AGAGTTTGATCMTGGCTCAG and 1492R GGYTACCTTGTTACGACTT described by Tanner et al. (2000). The reference strain was *L. lactis* subsp. *lactis* (BCRC 10791). For the PCR identification, genomic DNA was extracted using the Genomic isolation kit (GeneMark, Georgin, USA) according to the manufacture's instructions. Genomic DNA concentration was determined spectrophotometrically (Hitachi, U-2800A, Tokyo, Japan). PCR primers were used to amplify a 1484 bp DNA fragment. The reaction mixture contained 10 µl genomic DNA, 2.5 units of *Taq* polymerase (Promega, Madision, WI), 2 µl each of 10 mM dATP, dTTP, dCTP and dGTP, 5 µl of 10 X reaction buffer (10 mM Tris-HCl (pH 8.3 at 25°C) containing 50 mM KCl, 0.01% Triton X-100, 0.01% gelatin, 6.0 mM $MgCl_2$), and 50 pmol of each primers in a final volume of 50 µl. The DNA was denatured at 94°C for 2 min and amplified for 35 cycles at 94°C for 40 s, 45°C for 50 s and 72°C for 50 s. A final extension incubation of 2 min at 72°C was included. Amplification reactions were performed on a thermal cycler (Perkin-Elmer GeneAmp PCR System 2400, Foster city, CA). The PCR products were purified with Gel/PCR DNA fragments extraction kit (Geneaid, Taipei, Taiwan) and sequenced by automated sequencing core laboratory, National Cheng Kung University (Tainan, Taiwan).

Estimation of bacteriocin activity

Overnight culture broth was heated at 70°C for 30 min to kill the cells and adjusted to pH 6, and stirred at 4°C for 4 h. Cells were collected by 20 min of centrifugation at 17000 x*g* , 4°C and washed

Table 1. Zones of growth inhibition of *S. mutans* by lactic acid bacteria.

Strains	Supernatant adjusted to pH 2.0	Supernatant adjusted to pH 4.0 and treated with catalase	Supernatant adjusted to pH 4.0 and treated with pepsin
	Inhibition zone (mm)		
MRS	15.0±0.2^{a}	10.0±0.0^{a}	10.0±0.1^{a}
BCRC10791	17.5±0.2^{b}	12.0±0.4^{b}	10.5±0.1^{b}
B0032	20.5±0.3^{d}	14.0±0.6^{c}	12.0±0.2^{d}
B0105	20.5±0.2^{d}	14.8±0.2^{d}	10.5±0.1^{b}
B0106	20.8±0.3^{de}	14.0±0.5^{c}	11.0±0.2^{c}
B0115	20.8±0.3^{de}	14.0±0.4^{c}	11.0±0.2^{c}
B0117	21.0±0.2^{e}	14.3±0.3^{cd}	12.0±0.2^{d}
B0125	20.0±0.1^{c}	14.5±0.4^{cd}	11.0±0.1^{c}
B0157	20.5±0.2^{d}	14.0±0.2^{c}	11.0±0.3^{c}
B0158	20.5±0.2^{d}	14.8±0.3^{d}	11.0±0.2^{c}

The data values are expressed as the mean ± SD (n = 3). Values in a row with different superscript letters are significantly different (P<0.05).

twice with 0.1 volume of 5 mM sodium phosphate buffer (pH 6.0). The cells were resuspended in a 0.25 volume of 100 mM NaCl (pH 2.0) and stirred at 4°C for 4 h. The resultant samples were centrifuged at 17000 x*g*, 4°C for 20 min. After the pH of the supernatants had been adjusted to 4.0, the resultant samples were filtered through a 0.22 µm filter (Yang et al., 1992; Wu et al., 2004). The protein concentration (Lowry et al., 1951), and bacteriocin activities were determined.

Bacteriocinogenic activity of bacteriocin was quantified using microtiter plate assay (Rojo-Bezares et al., 2007). Each well of the microtiter plate contained: 90 µl of twofold-concentrated MRS broth, 100 µl of cell-free supernatants at serial double dilutions and 10 µl of the indicator *S. mutans* (10^5 c.f.u. ml^{-1}, as final concentration), respectively. Microtiter plate cultures were incubated for 12–24 h at 35°C after which growth inhibition of the indicator strain was measured by optical density at 595 nm (OD595) in a Bio-Rad microtiter reader (model 450, Bio-Rad Laboratories, Hercules, California). Half maximal inhibitory concentration (IC50) was defined as the concentration of a bacteriocin that is required for 50% inhibition *in vitro* (50% of the OD595 of the positive growth control).

SDS-PAGE analysis and identification of the activity band

The molecular weight of pH purified bacteriocin preparations were determined by Tris-Tricine SDS-PAGE with 12% acrylamide resolving gel and 10% acrylamide spacer gel (Schagger and Von Jagow, 1987). The pH purified bacteriocin preparations along with low molecular weight markers (Sigma) were electrophoresed together in one gel at 20 mA for the first 2 h and then at 30 mA for another 12 h. Half of the gel was stained with Coomassie brilliant blue R-250 for molecular weight determination, meanwhile the another half of the gel was used for antibacterial activity assay by washing in sterile water, and overlaying with TSB agar plate seeded with 1% *S. mutans* in a Petri plate. The plate was incubated at 35°C for 24 h (Gao et al., 2010).

Characterization of bacteriocin

(A) Stability after different temperature and extreme pH treatments: pH purified bacteriocin preparations were incubated, respectively, in either thermostatic water bath at 80 and 100°C for 20, 40 and 60 min or in autoclave at 121°C for 15 min. By adjusting the pH value in a range of 2.0 to 11.0 and maintaining the bacteriocins for 2 h, the effect of pH was tested. For all the experiments described here, *S. mutans* was used as indicator and controls were maintained without any treatment (Gao et al., 2010).

(B) Sensitivity to proteolytic enzymes: The following enzymes were assayed (Rojo-Bezares et al., 2007): pepsin, protease, pronase, α-chymotrysin, bromelain and ficin (5 mg ml^{-1}) (all of them were purchased from Sigma, St. Louis, Missouri). Under conditions recommended by the manufacturer, antibacterial activity was assayed. For all the experiments, *S. mutans* was used as indicator and controls were maintained without any treatment. The percentage of inhibition activity was determined as: [(diameter of inhibition zone)^2 - (10 mm)^2]/[(diameter of maximum inhibition zone)^2 – (10 mm)^2] x 100%.

RESULTS

The antibacterial activity of LAB isolates

In this work, presumptive strains were first determined by phenotypical and physiological tests including Gram staining, catalase test and acid production. Acid-producing bacteria isolated from fermented mustard were cultivated in MRS broth and the activities of spent cell supernatants (SCS) against *S. mutans* using agar well diffusion test method were obtained (Table 1). The effect of low pH and hydrogen peroxide were eliminated by adjusting pH and catalase hydrolysis, respectively. These isolates with antibacterial activity against *S. mutans* were selected for the further experiments. After treatment by pepsin, the antibacterial activity of SCS of test strains, especially for B0105 and BCRC10791, almost disappeared, indicates that the substance with antibacterial activity was sensitive to pepsin.

Strain identification

Amplification of 16S rDNA sequence by PCR was performed using the primers described by Tanner et al. (2000). For 16S rDNA molecular identification of strain B0105, 1496 bp fragment was amplified from its genomic

DNA and the nucleotide sequence was determined. It revealed that the 16S rDNA nucleotide sequence of strain B0105 was 100% identical with that of *L. plantarum* strain WCFS1. Thus, strain B0105 was identified as *L. plantarum* and the GenBank access number is AL935258 was assigned.

Extraction of adsorbed bacteriocin and determination of its molecular weight

Bacteriocin-like compounds were adsorbed at pH 6.0 to B0105 and the cells were then centrifuged and resuspended in 100 mM NaCl at pH 2.0 for 4 h to release the bacteriocin-like compounds from the cell of B0105. The protein concentrations of released bacteriocin-like and activity was determined. The protein concentration of released bacteriocin-like compound was 867.9 µg ml^{-1} and was used to inhibit the indicator strain. According to the results from Table 2, pH purified bacteriocin-like compound significantly inhibited the growth of *S. mutans*. The IC_{50} ranged from 7.81-15.62 µg ml^{-1}.

According to the Tris-tricine-SDS–PAGE analysis, bacteriocins-like B0105 are small polypeptides with a molecular weight in the range of 3.5 and 4.7 kDa (Figure 1A). Interestingly, after Tris-tricine-SDS–PAGE, the bands of bacteriocin B0105 were still active against the indicator strain *S. mutans* (Figure 1B).

Characterization of bacteriocin

The bacteriocin B0105 treated with pH and its antibacterial activity was determined with agar-well diffusion test against *S. mutans* (Table 2). The antibacterial activity of bacteriocin B0105 against *S. mutans* decreased with increased pH values. No antibacterial activity was obtained when above 6.0. Table 3 also shows that the characteristics of the bacteriocin-like compound were stable after it was treated at 100°C for 1 h with 84.5% of antibacterial activity remaining. As far as proteases were concerned, the antibacterial activity of the bacteriocin-like compound was decreased when it was treated with different proteases, including trypsin, a-chymotrypsin, pronase, protease, bromelain and ficin.

DISCUSSION

It was observed that fermented vegetables or fruits are good sources of LAB. Many bacteriocin-producing LAB such as Lactobacillus sake C2, *L. plantarum* C19, *Lactobacillus brevis* P-319 and *Lactobacillus sakei* KTU05-6, *Pediococcus acidilactici* KTU05-7, *Pediococcus pentosaceus* KTU05-8, KTU05-9 and KTU05-10 strains, showing activity towards various indicator strains, were successively isolated from fermented vegetables (Gao et al., 2010; Delgado et al., 2005; Li et al., 2008; Cizeikiene et al., 2013). Similiarity, strain B0105 was isolated from fermented vegetables and identified as *L. plantarum*. In this study, the antibacterial activity of B0105 and B0158 isolates against *S. mutans* was maintained after adjustment of pH and removal of catalase, indicating that the antibacterial agents of B0105 and B0158 could be bacteriocin-like compounds. After treatment with pepsin, no antibacterial activity of B0105 was obtained, indicating that bacteriocin-like compound is peptide (Rojo-Bezares et al., 2007). In another report, the bactericidal effect might be from the production of organic acids and/or in combination with the production of bacteriocin (Lin et al., 2008).

Adsorption of the bacteriocins onto cells was strongly influenced by the pH of the suspending environment. Maximum adsorption of nisin to both producer and indicator bacteria occurred at pH 6.5, showed complete loss of adsorption at pH 3.0 and below (Yang et al., 1992). Yildirimi et al. (2002) also noted that the adsorption of buchnericin LB was maximal between pH5.0 and 8.0 (100%) but below or above these values, the adsorption was decreased to 50%. The effects of pH value on isolation of bacteriocin-like B0105 was adsorbed at pH 6.0 to B0105, and then the cells were resuspended in pH 2.0 to release the bacteriocin from the cell of B0105. Extraction at alkaline pH was not used because like nisin, pediocin AcH, and some other bacteriocins of lactic acid bacteria are inactivated at alkaline pH (Yang et al*.,* 1992). It has been reported that *L. acidophilus* produced a acidocin B, it retained 50% activity at pH 5.0 (Han et al., 2007). Lin et al. (2008) also indicated that cell cultures of LAP5 strain were neturalized to pH 7.0, the antagonistic effects of LAP5 against the *Salmonella* growth showed no inhibitory activity. Therefore, the pH ranges of antibacterial activities of B0105 was pH 2.0 to 6.0, the inhibition activity to *S. mutans* became negligible at pH 7.0 or above. Rojo-Bezares et al. (2007) reported that the MIC50 value of nisin against *Oenococcus oeni* was 0.024 and 12.5 µg ml^{-1} for other wine LAB species. Our results also revealed bacteriocin B0105 as an efficient antibac-terial agent again *S. mutans* with IC_{50} from 7.81-15.62 µg m^{-1}.

According to the Tris-tricine-SDS–PAGE analysis, bacteriocin-like B0105 are small polypeptides with a molecular weight in the range of 3.5 and 4.7 kDa. However, the range of bacteriocins for *L. plantarum*, *plantaricin* ST31 (2.76Da) from *L. plantarum* ST31 (Todorov et al., 1999), bacST202Ch and bacST216Ch (3.5 and 10.0 kDa) from *L. plantarum* (Todorov et al., 2010), bacteriocins ST28MS and ST26MS (5.5 and 2.8 kDa) from *L. plantarum* (Todorov and Dicks, 2005) are also reported. The results of measured molecular weight of bacteriocin B0105 suggested that its molecular weight is almost the same as the reports. The bacteriocins of LAB against LAB, Gram positive or other pathogenic bacteria were studied (Elegado et al*.,* 2004; Yildirim et al*.,* 2002; Lin et al., 2008; Gao et al., 2010), but *S. mutans* was not included in their study.

It is well known that heat stability of bacteriocins are important if they are to be used as food preservative agent, because many procedures of food preparation involve

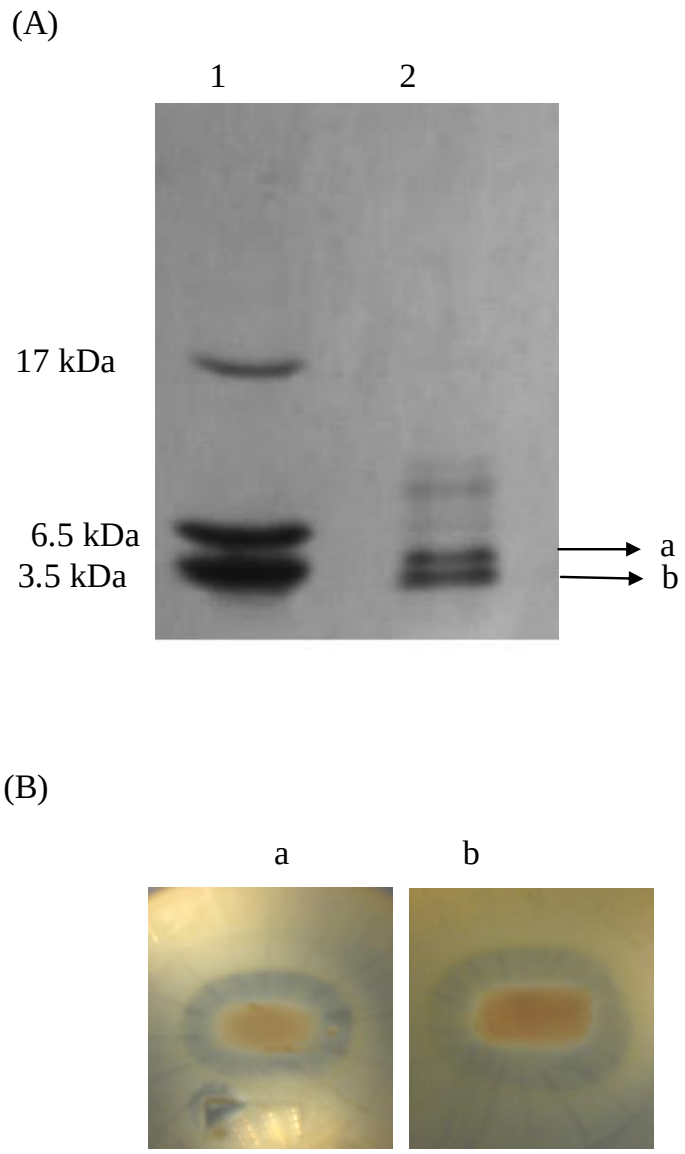

Figure 1. (A) A bacteriocin profile of SDS-PAGE of *L. plantarum* B0105. Lane 1: Ultra-low range molecular weight marker; Lane 2: Bacteriocin of *L. plantarum* B0105; (B) The gel band a, b overlaid with Tryptic soy soft agar surface seeded with 1% *S. mutans*.

Table 2. Antibacterial activity of extracted bacteriocin against *S. mutans* at different protein concentration.

Protein concentration (µg/ml)	Bacterial growth (OD_{595})		
	12 h	18 h	24 h
Control[a]	0.667±0.010[a]	0.669±0.012[a]	0.678±0.014[a]
250	0.054±0.011[b]	0.074±0.008[b]	0.085±0.009[b]
62.25	0.134±0.008[c]	0.139±0.018[c]	0.141±0.015[b]
15.62	0.351±0.020[d]	0.335±0.016[d]	0.328±0.018[ab]
7.81	0.348±0.028[d]	0.358±0.037[d]	0.348±0.037[ab]

Control[a]: containing *S. mutans* but no bacteriocins; The data values are expressed as the mean ± SD (n = 3). Values in a row with different superscript letters are significantly different (P<0.05).

Table 3. Effect of pH, heat and proteolytic enzyme on the antibacterial activity of bacteriocins from *L. plantarum* against *S. mutans*.

Treatment	Inhibition zone (mm)/Inhibition activity (%)[a]
pH	
2.0	20.0 ±0.0[a]/(100)
3.0	17.8±0.1[b]/(72.3)
4.0	16.0±0.1[c]/(52.0)
5.0	13.3±0.1[d]/(25.6)
6.0	11.5±0.1[e]/10.8)
7.0	10.0±0.0[f]/(0)
8.0	10.0±0.0[f]/(0)
9.0	10.0±0.0[f]/(0)
10.0	10.0±0.0[f]/(0)
11.0	10.0±0.0[f]/(0)
Heat	
80°C, 20 min	20.0±0.0[a]/(100)
80°C, 40 min	20.0±0.0[a]/(100)
80°C, 60 min	20.0±0.0[a]/(100)
100°C, 20 min	20.0±0.0[a]/(100)
100°C, 40 min	20.0±0.0[a]/(100)
100°C, 60 min	20.00.0[a]/±(100)
121°C, 15 min	18.8±0.1[b]/(84.5)
Proteolytic enzyme	
control	20.0±0.0[a]/(100)
pepsin	14.0 ±0.1[b]/(34.4)
protease	15.5±0.1[c]/(50.0)
pronase	14.5±0.1[d](39.3)
α-chymotrysin	14.5±0.1[d]/(39.3)
bromelain	14.0±0.1[b](44.6)
ficin	15.0±0.1[f]/(29.8%)

[a]Inhibition activity (%) = [(diameter of inhibition zone) ^{2} – (10 mm) ^{2}/(diameter of maximum inhibition zone) ^{2} – (10 mm) ^{2}] × 100%. *The data values are expressed as the mean ± SD (n = 3). Values in a row with different superscript letters are significantly different (P<0.05).

heat treatment. Major classes of bacteriocins produced by LAB include lantibiotics (class I) and large heat-labile protein (class III) and complex proteins (class IV) whose activity requires the association of carbohydrate or lipid moieties (Llaenhammer, 1993). In this study, the antibacterial activity of bacteriocin-like B0105 treated at 121°C for 15 min was 84.5% against *S. mutans*. Consequently, bacteriocin-like B0105 has a stable behavior similar to other bacteriocin reported (Rojo-Bezares et al., 2007; Han et al., 2007; Yildirim et al., 2002). In other words, bacteriocin-like B0105 has considerable heat and pH tolerance which could make it useful additive in foods.

Conclusions

Traditional fermented mustard is a rich source of probiotic LAB. From isolated LAB strains, strain B0105 which produced a bacteriocin-like compound which strongly inhibited *S. mutans* was isolated from traditional fermented mustard. This strain was identified as *L. plantarum* by phenotypical, physiological tests and 16S rDNA sequence. This may be the first bacteriocin-producing strain of *L. plantarum* against *S. mutans*. After extraction by cell adsorption-desorption, the molecular weight of bacteriocin B0105 was 3.5 and 4.7 kDa in Tris-tricine SDS-PAGE. After Tris-tricine-SDS-PAGE, the bands of bacteriocin were still active against the indicator strain *S. mutans*. Bacteriocin-like compounds produced from B0105 had the strongest antibacterial activity and exhibited heat stability, pH stability and sensitivity to proteolytic enzyme. The present study recommended that bacateriocins produced from strain B0105 may be a potential candidate for development as an antibacterial agent. Further work would be done to determine the sequence of bacteriocin gene and ascertain the mode of action of bacteriocin and investigate the effects of bacteriocin B0105 on food

quality.

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Full Length Research Paper

Research on biological aerated filter with volcanic filler for pretreatment of micro-polluted source water in lower temperature

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A pilot-scale research on dealing with micro-polluted source water of 4.2 m^3/h by biological aerated filter (BAF) with volcanic filler for pretreatment was carried out. Under the experimental condition of air/water ratio 1:1, and the ammonia nitrogen of raw water 1.57 to 2.72 mg/L, the ammonia nitrogen average removal rate was 95.8%, when water temperature was above 10°C; and the removal rate was still above 70%, when water temperature ranged from 5.5 to 10°C. At the same time, there was no accumulation of nitrite nitrogen in the reactor. Moreover, the removal rate of Mn, COD_{Mn} and UV_{254} was 66.3, 14.1 and 3.2%, respectively. Backwashing process of the BAF had little influence on the biofilm, which could be recovered within 1 to 2 h after the backwashing. The experimental result show that the removal effect of ammonia nitrogen and organic matter was very significant in BAF, even in lower temperature, which could guarantee the treatment effect of the follow-up processing unit.

Key words: Volcanic filler, biological aerated filter, biological pretreatment, micro-polluted water, lower temperature.

INTRODUCTION

With the development of industry and their drastic growth, drinking water sources are polluted heavily. Most sources cannot meet the standard of drinking quality in China, especially in south China. The pollutant of micro-polluted source water was very complicated and serious, especially ammonia nitrogen (NH_4^+-N) and organic matter, which could not be effectively removed by conventional process, and the effluent of conventional process could hardly reach the drinking water standards in China (GB/T5750-2006).

Biological pretreatment technology had been the research focus of the water treatment (Yu et al., 2003; Li et al., 2006, 2007; Xu et al., 2002), since conventional process could not remove ammonia nitrogen and organic matter sufficiently. Water treatment by biological methods were known to be environment friendly and be demonstrated with little byproduct. Therefore, pretreatment of polluted source water by biological methods should be a trend for drinking water treatment. Biological aerated filer (BAF) was one of the favorite biological methods in the field of water treatment, and could effectively reduce ammonium and organic matter through microbe mechanisms (Han et al., 2012). BAF has many advantages, such as small volume, little covering earth and hydraulic retention time (HRT), high treatment efficiency and effluent water quality, low investment and running cost etc. Today, BAF has been widely studied and applied in wastewater treatment, and some other biological filtration methods had been successfully researched for drinking water treatment, but limited studies were reported to lend BAF as a pre-treatment method for drinking water production.

In this paper, a pilot-scale research on dealing with micro-polluted source water of 4.2 m^3/h by biological aera-

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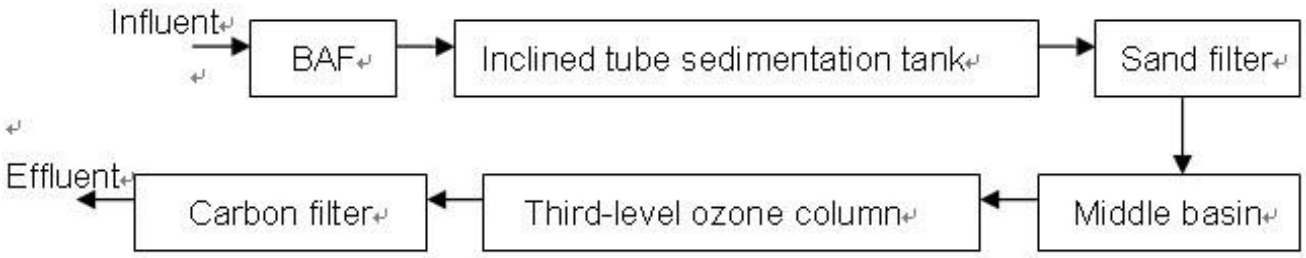


Figure 1. The pilot system process.

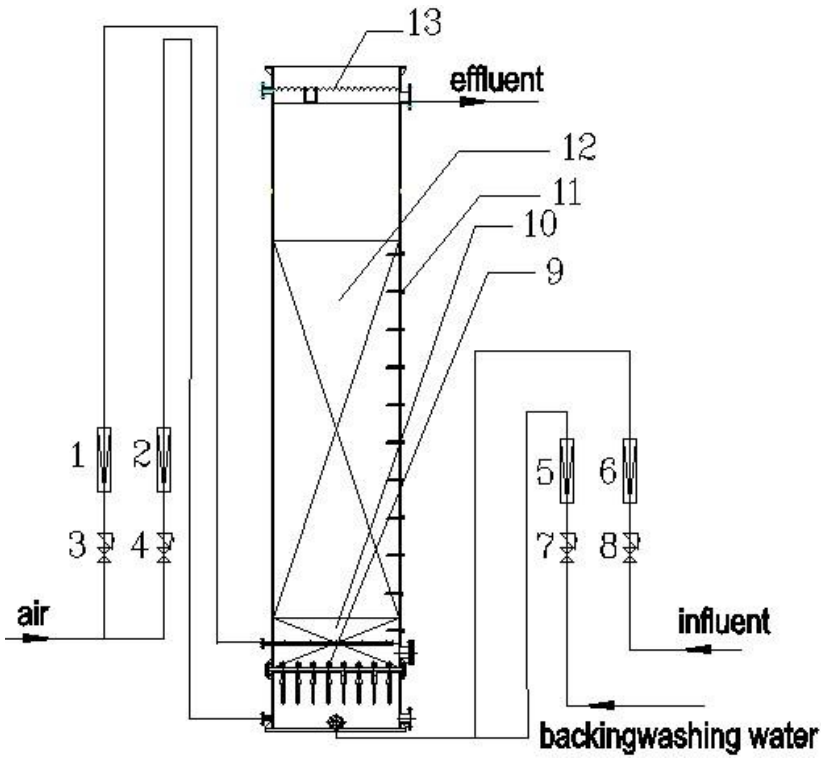


Figure 2. Schematic diagram of the experimental procedure of BAF.1. Backwashing air glass rotameter; 2. Aeration rotameter; 3~4. Solenoid valve; 5. Backwashing water rotameter; 6. Influent electromagnetic flowmeter; 7. Pneumatic valve; 8.Ball valve; 9. Long-handled filter head; 10. Graded gracel layer; 11. Sampling port; 12. filter layer; 13. Sink.

ted filter (BAF) with volcanic filler for pretreatment was carried out in Guanjinggang Water Supply Plant in Jiaxing, China. The experimental data showed that biological aerated filter (BAF) with volcanic filler was a promising means to pretreat ammonia nitrogen and organic matter in micro-polluted source water, even in lower temperature.

MATERIALS AND METHODS

The pilot system process

The pilot system process is shown in Figure 1. The biological pretreatment and the conventional treatment process and deep processing of the water treatment was carried out in the pilot, dealing with micro-polluted source water of 4.2 m^3/h. The role of BAF as pretreatment unit was mainly studied in this paper.

BAF experimental device

The testing device is shown in Figure 2.

Raw water

The experimental raw water was sampled from the influent of Guanjinggang Water Supply Plant in Jiaxing, China. Table 1 summarizes the main parameters of the raw water.

Table 1. Raw Water Quality and Parameter.

Item (unit)	Variation range	Mean
Turbidity (NTU)	14.2-51.2	28.5
COD_{Mn}(mg/L)	4.69-6.27	5.58
NH_4^+-N(mg/L)	0.77-4.13	2.11
nitrite(mg/L)	0.091-0.31	0.16
nitrate(mg/L)	1.52-3.38	2.67
Fe(mg/L)	0.52-2.98	1.73
Mn(mg/L)	0.201-0.608	0.327

Table 2. Analytical Methods.

Item	Analytical method
Temperature (°C)	Thermometer
Turbidity (NTU)	Portable turbidity meter
NH_4^+-N (mg/L)	Spectrophotometry
COD_{Mn} (mg/L)	Acid potassium permanganate titration method
Fe (mg/L)	Spectrophotometry
Mn (mg/L)	Spectrophotometric
Nitrate (mg/L)	Spectrophotometry
Nitrite (mg/L)	Spectrophotometry
UV_{254} (mg/L)	UV spectrophotometer

The main test items and analytical methods

The water quality analysis method was the Standard Examination Methods for Drinking Water (GB/T5750-2006), and specific methods are shown in Table 2.

RESULTS AND DISCUSSION

Earlier than this experiment, BAF had operated for about 10 months, so the biofilm was mature. During the experimental period, the conditions were as follows: air/water ratio of 1:1, raw water temperature of 5.5 to16°C, and the filter velocity of 5.5m/h.

The removal effect of turbidity

As shown in Figure 3, the average removal rate of turbidity was 20.6%, when the raw water turbidity ranged from 14.2 to 51.2 NTU. The turbidity of raw water was removed by physical interception of filter layer and the adsorption and flocculation of biofilm (Yu, 2002; Zhang and Liu, 2005). The experiment data showed that the turbidity removal rate of raw water was lower, because up-flow method was adopted in the experiment, which could not remove turbidity effectively. But removing turbidity was not the main purpose of BAF, and the turbidity could be removed in coagulation and sedimentation unit. In addition, when the turbidity removal rate was lower, the probability of suspended solids (SS) covering on biofilm would be reduced. In this case, the nitrifying bacteria biofilm was active so that the ammonia nitrogen nitrification was maintained at a high level. Besides, the decline of turbidity removal rate meant lower speed of filtration loss headed to prolong backwashing cycle.

The removal effect of ammonia nitrogen

As shown in Figure 4, when the experimental condition was air/water ratio of 1:1, the ammonia nitrogen of raw water ranged from 1.57 to 2.72 mg/L with an average of 2.11 mg/L, the ammonia nitrogen average removal rate was 95.8%, when water temperature was above 10°C; and the removal rate was still above 70%, when water temperature ranged from 5.5~10°C. Biofilm could be easily formed on the volcanic filter because of its unique features, such as rough surface, large specific surface area and high porosity. The volcanic filter provided an ideal growth and breeding condition for micro-organisms. So the ammonia nitrogen was effectively removed by the oxidation of nitrifying bacteria even in lower temperature due to the volcanic filler providing prodigious biofilm.

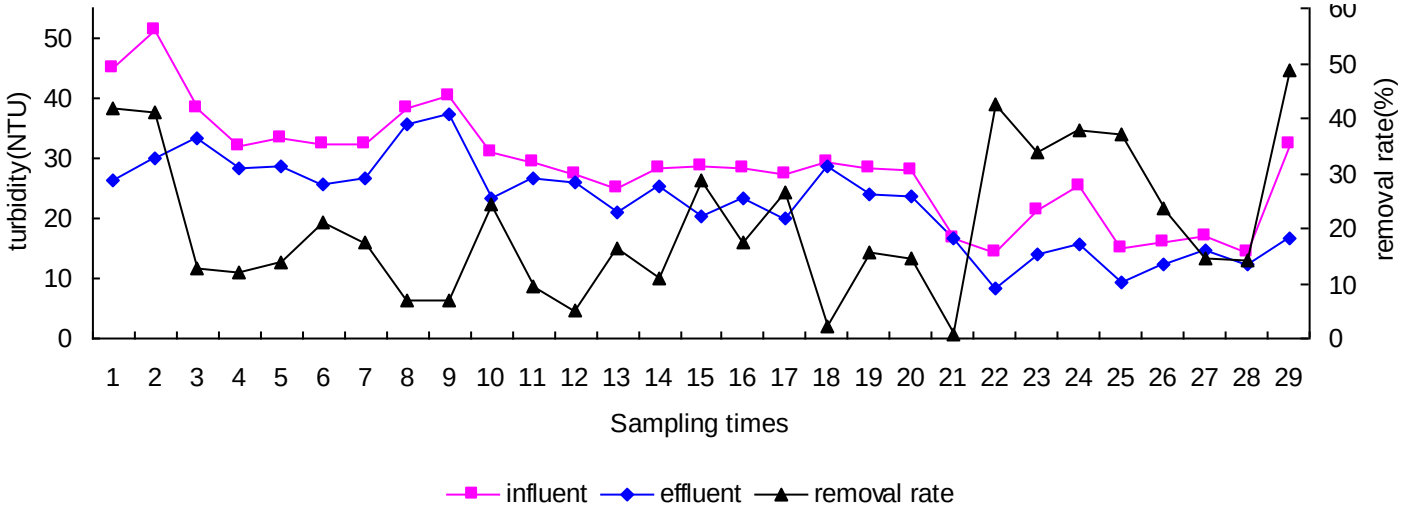


Figure 3. Turbidity removal effect in BAF.

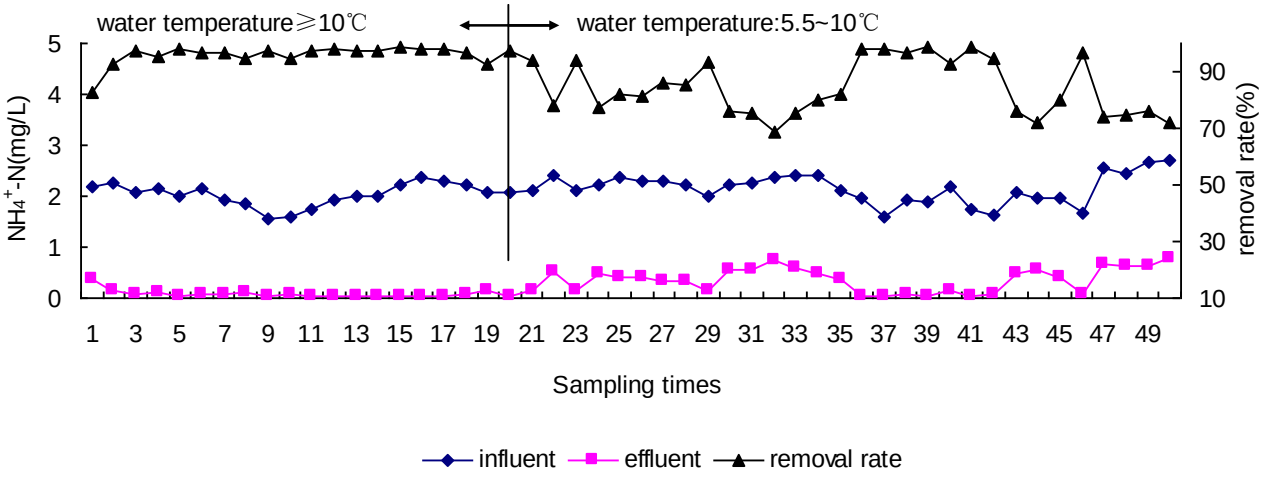


Figure 4. Ammonia nitrogen (NH4+-N) removal effect in BAF.

The removal effect of nitrite

As shown in Figure 5, under the experimental condition that the air/water ratio was 1:1, the raw water nitrite was 0.091 to 0.31mg/L, and the average removal rate of nitrite nitrogen in BAF was 65.5%, when the temperature ranged from 5.5 to 16°C. There was no accumulation of nitrite in the reactor, so BAF had an excellent ability to remove nitrite. The experimental results show that nitrate nitrogen of the effluent increased to 1.75 mg/L; when ammonia of the raw water ranged from 1.57 to 2.72 mg/L, nitrite of the raw water was 0.091~0.31mg/L. The experimental data showed that BAF had strong nitrification ability.

The removal effect of COD_{Mn}

As shown in Figure 6, under the experimental condition that the air/water ratio was 1:1, the raw water COD_{Mn} ranged from 4.69 to 6.27 mg/L with an average of 5.58 mg/L; the average removal rate of COD_{Mn} was 14.13%. The result shows that BAF as pretreatment had positive effect on conventional processing because it not only partly intercepted organic particles but also degraded dissolving organic matter which can hardly be removed by conventional processing.

The removal effect of Mn

As shown in Figure 7, under the experimental condition that the air/water ratio was 1:1, the raw water Mn ranged from 0.201 to 0.327 mg/L, and the average removal rate of Mn was 66.3%, when the water temperature ranged from 5.5~16 °C. The oxidation of Mn was carried out with the catalytic action of bacteria extracellular enzyme, and Mn could be effectively removed when the microbial biomass reached a certain extent. Theoretically, the oxida-

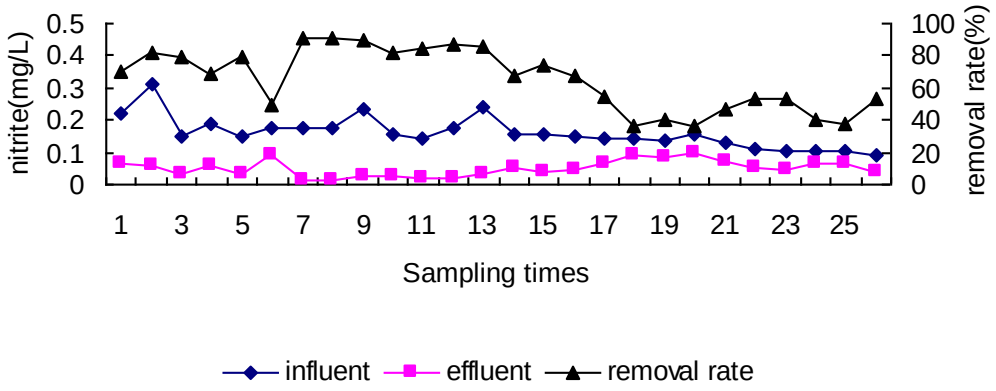


Figure 5. Nitrite removal effect in BAF.

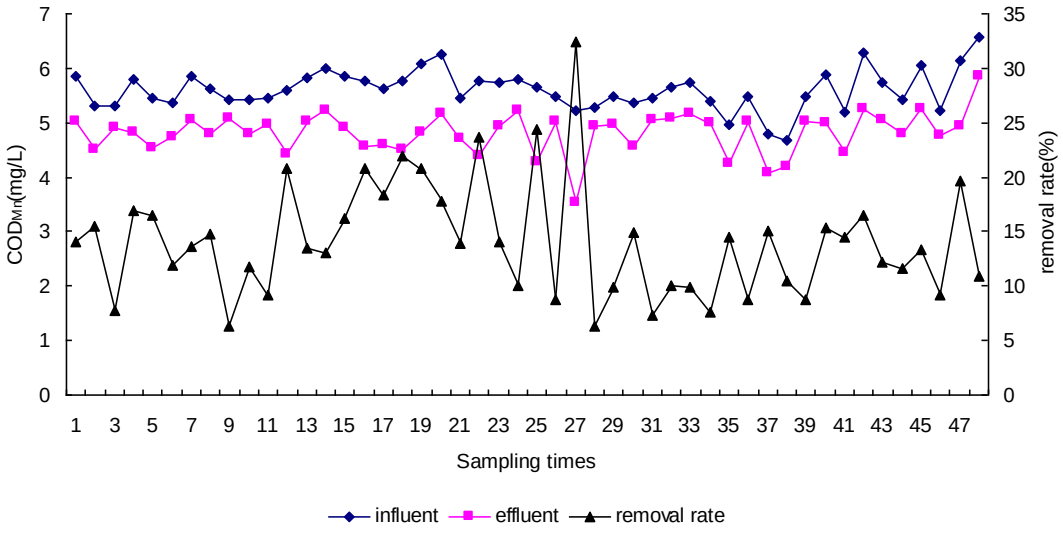


Figure 6. CODMn removal effect in BAF.

tion process of Mn begun after the oxidation process of Fe (Yang et al., 2003). In this experiment, the average removal rate of Fe was 21.9%, and the concentration of Fe of effluent was 1.15 mg/L, therefore, Mn could not be removed completely; maybe the Fe was not oxygenated incompletely.

The removal effect of UV254

The UV254 removal rate ranged from 0 to 8% with an average of 3.42%, when the water temperature ranged from 5.5~16°C. The removal rate of UV_{254} of raw water in BAF was lower, so the removal rate of haloform precursor's elimination of raw water in BAF, which had the good relevance with UV254 was lower.

The influence of backwashing on BAF

Backwashing was carried out when BAF performance began to decline. The backwashing cycle was 7 to 14 days, with air strength of 10 to 15 L/(m^2·s) for 10 min, and then with water of the strength of 10 L/(m^2·s) for 10 min. After the backwashing, the effluent of BAF was sampled for 1 to 2 h for testing; the removal rate of ammonia was above 80% (Figure 8), and the removal rate of COD_{Mn} was also above 14%. The result shows that the suitable backwash intensity and time would not affect the treatment of BAF.

Microscopic observation of the media

We removed the carrier samples from the filter layer at the different depths along the flow direction, respectively. After the samples were pretreated according to the requirements, they were observed by scanning electron microscope (SEM). The samples were taken from the inflow 0.3, 1.6, 2.4m, respectively. Scanning electron microscopy showed that the biomass of the carrier decreased significantly with the filter layer depth. On the carrier of filter layer 30 cm (Figure 9b), there were growth

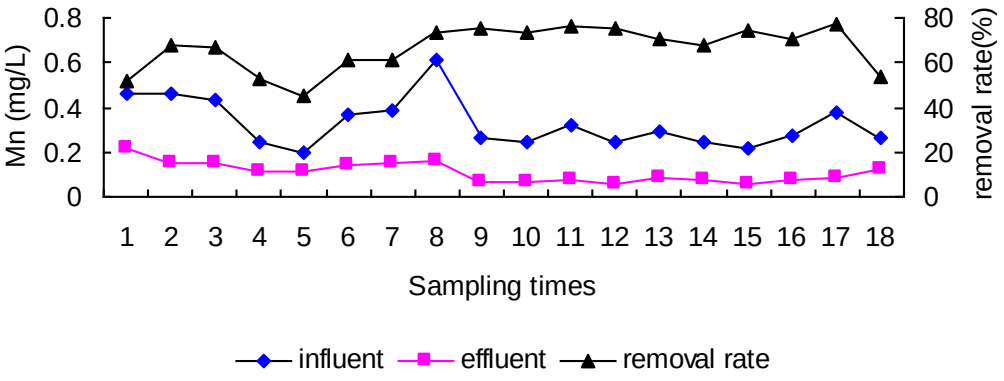


Figure 7. Mn removal effect in BAF.

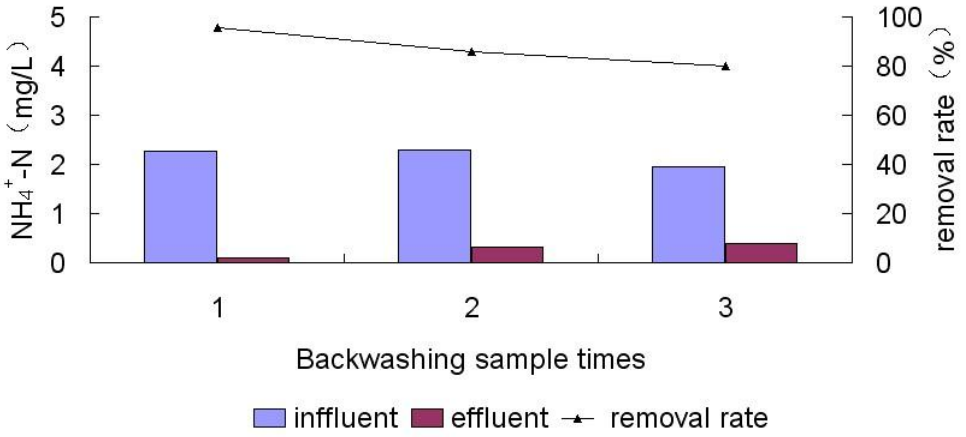
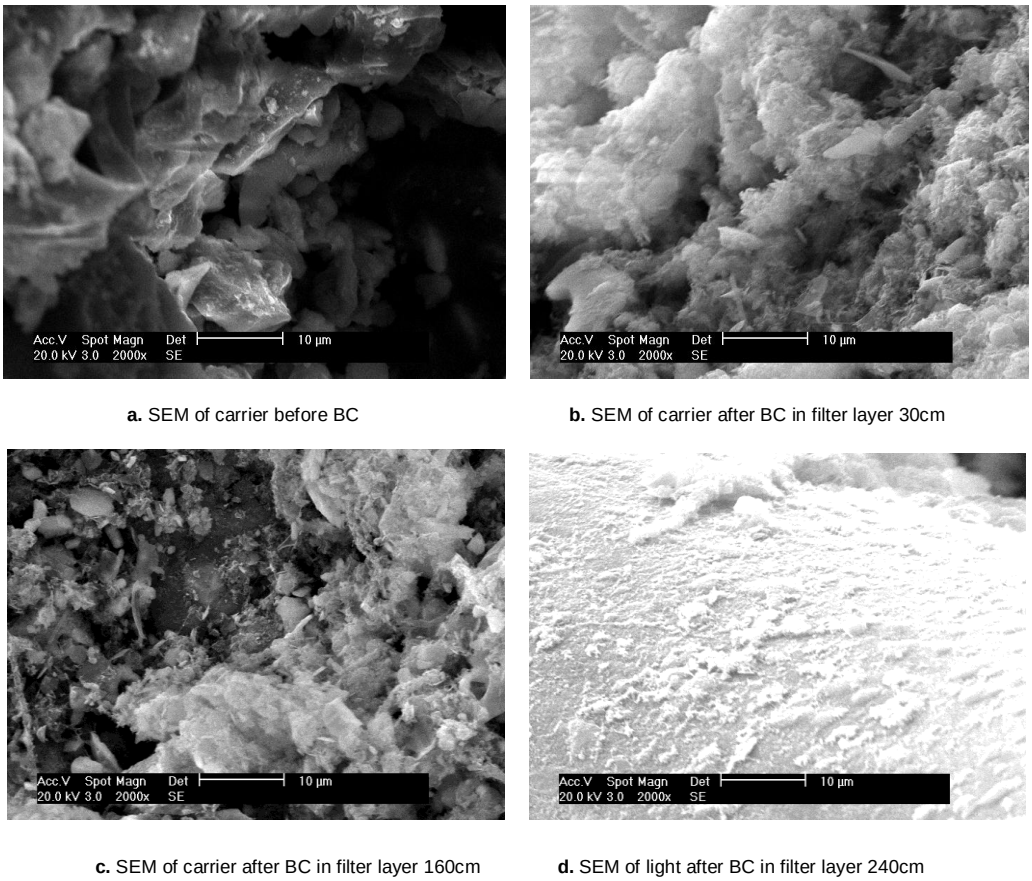


Figure 8. The influence of backwashing on BAF.

of more microorganisms on the carrier sunken place, and it was covered by a large number of filamentous fungi, filamentous fungi crisscross, forming biofilm skeleton, such as aureus and bacillus inclusion in it, and in addition, there were a few very fine filamentous cell secretion. Bacterial surface adsorbed a great number of inorganic or organic debris due to the reason that the test source water turbidity was higher; so many filamentous fungi, bacteria and bacilli were coated. The biofilm structure was not dense, there are many large gaps, and this was conducive to the matrix and dissolved oxygen mass transfer. The filamentous fungi at carrier of filter layer 160 cm was significantly reduced (Figure 9c) and there was a few filamentous fungi in the 240cm outflow (Figure 9d). This shows that the microbial groups and quantity were reduced as the filter layer depth increased. Biofilm could not completely cover the surface of the carrier when observed by scanning electron microscope. There was usually only scattered individual cell or a few cells together in the carrier surface trunk and smooth place, on the contrary, a great number of microorganisms grew in the sunken place or holes; the growth thickness highly correlated the depth of the sag. This shows that surface depression and holes were easy adherent microbes by microorganisms. Carrier surface roughness had great influence with biomass and the biofilm formation velocity. Unlike wastewater treatment, the biofilm at carriers was very thin in BAF in poor nutrient micro-polluted source water treatment.

Conclusions

Under the experimental condition that the air/water ratio was 1:1, the ammonia nitrogen of raw water ranged from 1.57 to 2.72 mg/L, and the ammonia nitrogen average removal rate was 95.8%, when water temperature is above 10 °C; and the removal rate was still above 70%, when water temperature ranged from 5.5 to 10 °C. The data showed that BAF with volcanic filler as pretreatment of micro-polluted source water could effectively remove ammonia nitrogen. The removal rate of Mn, COD_{Mn} and UV_{254} were 66.3, 14.1 and 3.2%, respectively. The suitable backwashing process of the BAF had little influence



a. SEM of carrier before BC

b. SEM of carrier after BC in filter layer 30cm

c. SEM of carrier after BC in filter layer 160cm

d. SEM of light after BC in filter layer 240cm

Figure 9. Comparison of the carriers before and after biofilm culturing (BC). A, SEM of carrier before BC; B, SEM of carrier after BC in filter layer 30 cm; C, SEM of carrier after BC in filter layer 160 cm; D, SEM of light after BC in filter layer 240 cm.

on the biofilm, which could be recovered within 1 to 2 h after the backwashing. Biofilm could not completely cover the surface of the carrier, and microorganisms mainly grew in the sunken place or holes. For micro-polluted source water, BAF with volcanic filler was a promising means to pretreat ammonia nitrogen and organic matter, even in lower temperature.

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Full Length Research Paper

Comparative clinical study on diagnostic detection of hepatitis E virus between nested polymerase chain reaction (PCR) and serological tests

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This study was carried out to evaluate the diagnostic value of nested reverse transcriptase-polymerase chain reaction (RT-PCR) for HEV (hepatitis E virus) RNA detection relative to anti-HEV immunoglobulin G (IgG) and IgM enzyme-linked immunosorbent assays. One hundred and twenty six (126) patients with sporadic acute hepatitis E were included in this study. We focused on the popular genotype I and IV of HEV-positive patients in China, and selected the conserved region located in ORF2 for designing a set of nested RT-PCR primers. HEV RNA was detected in 53.2% (67/126) of patients, while all control subjects were negative for HEV RNA. The total agreement between IgM and nested RT-PCR detection was 80.9%, showing a fine coincidence. The results further suggested that there was a significant difference between nested RT-PCR detection and IgM ELISA: 3 cases with positive results for HEV RNA showed negative anti-HEV IgM at the early phrase, and presented positive IgM reaction in succession after the trail of detection. HEV RNA was detected in serum samples from sporadic acute hepatitis patient usually by day 1 to 12 after the onset of symptoms, but showed a decreasing sensitivity with the increasing disease course. From these experiments, we can conclude that HEV RNA detection is of great clinical significance, which has an obvious advantage in diagnosis of early infection of HEV.

Key words: Hepatitis E virus, nested reverse transcriptase-polymerase chain reaction (RT-PCR), RNA, antibody.

INTRODUCTION

Hepatitis E (HE) remains an important public health problem in developing countries, and is diagnosed in some developed countries with sporadic cases (Emerson and Purcell, 2003). The HEV (hepatitis E virus) target population is young to middle aged adults, 15 to 40 years of age. The clinical symptoms are typical of acute viral hepatitis that includes jaundice, malaise, anorexia, nausea, abdominal pain, fever and hepatomegaly. The disease is self-limiting and generally no chronic sequelae has been reported (Emerson and Purcell, 2003). Increased morbidity and mortality is observed in chronic liver disease patients superinfected with HEV. A unique clinical feature is its increased incidence and severity in pregnant women, with mortality rates of 15 to 20% (Kumar et al., 2004). Therefore, research on the discovery and validation of biomarkers of hepatitis E patients and their association with disease severity would be important.

Hepatitis E virus (HEV) is a non-enveloped RNA virus and its genome is a single-stranded, positive-sense RNA of 7.2 kb. It contains a short 5'-untranslated region (5'-UTR) followed by three ORFs (ORF1, ORF2 and ORF3)

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and then a short 3'-UTR with a poly(A) tail. The ORF2 of HEV encodes its capsid protein (pORF2) of 660 amino acids and is proposed to encapsidate the viral RNA genome (Chandra et al., 2008). Based on nucleotide sequence analysis, mammalian HEV has been divided into four genotypes, namely genotypes 1to 4. Genotype 1 is responsible for the majority of HEV infections in developing countries; genotype 2 consists of strains not only in Mexico but also in African countries including Chad, Namibia and Nigeria; genotype 3 is widely distributed throughout the world except in Africa; and genotype 4 is distributed exclusively in Asian countries. In China, genotypes 1 and 4 have become the dominant cause of HE (Zhu et al., 2011; Tai et al., 2009). Recently, detection of serum IgM specific for HEV by enzyme-linked immunosorbent assay (ELISA) is the most popular method used for the diagnosis of hepatitis E, but with lower sensitivity and missing cases with HEV infection (Favorov et al., 1994). Since various kinds of factors could influence the stability of the detection, including HEV window phase, patient autoimmune status and the specificity of tests, it is necessary to develop another assay with higher diagnostic value for HEV detection. The aim of the present study was to detect HEV RNA in serum using nested reverse transcriptase (RT)-PCR and to evaluate this detection for clinical diagnosis of hepatitis E in comparison with the well-adopted ELISA assay.

MATERIALS AND METHODS

Sample collection

A total of 126 serum samples were randomly collected from 34 patients with sporadic acute hepatitis E in Guangzhou, Guangdong, China. Twenty four of them were sampled for 4 times during the treatment, and the other 10 of them were sampled for three times during the treatment. Besides, 20 serum samples from patients with hepatitis A, 30 from patients with hepatitis B and 30 hepatitis C serum samples were collected for comparison. Thirty healthy volunteers were included in the study as controls. After centrifugation at 3500 rpm for 10 min, sera was separated and stored immediately in a -80°C freezer until use.

Detection of serum HEV RNA by nested RT-PCR

Since only genotypes 1 and 4 of HEV were causing pandemic in China, we focused on genomic sequences of genotypes I (EMBL ID: D11092, L08816, L25547, M94177) and IV (EMBL ID: AB108537, AJ 272108) and selected the conserved region located in ORF2 for designing a set of nested RT-PCR primers. RT-PCR was performed using a QIAGEN One-Step RT-PCR kit (QIAGEN) according to the manufacturer's instructions. cDNA was produced in a reaction tube containing 10 µL of the template RNA, 4 µL of the 5×QIAGEN One-Step RT-PCR buffer, 0.2 µL primer (50 mmol L^{-1}), 2 µL of the dNTP mix (containing 10 mM of each dNTP), 20 U of the RNase Out RNA inhibitor, 12 U of AMV reverse transcription enzyme mix, 2.3 µL of the RNase-free water. The thermal cycling conditions included one step of reverse transcription for 1 h at 42°C. The primers for nested PCR were as follows: the external forward primer M1[5'-AATTATGCCGAGTAACCGGGTT-3'] and reverse primer M2 [5'-CCCTTATCCTGCTGAGCATTCCC-3']. The reaction system in a volume of 50 µL contained 5 µL of 10×PCR buffer, 1 µL of each primer (M1, M2, M3 and M4) (50mmol L^{-1}), 1 µL of the dNTP mix (10 mM of each dNTP), 0.5 µL of Taq enzyme (5 U μL^{-1}), 5 µL of the RT-PCR product. The nested forward primer M3[5'-GTTATGTTTTGCATAGAAATGGC-3']), products from first round were used as the templates for the second round of nested PCR. The nested reverse primer M4 [5'-AGCCGACGAAATCAATTCTGTC-3']; house keeping gene β-actin: [sense-primer 5'-GTCGTACCACTGGCATTGTG-3', anti-sense 5'-CCATCTCTTGCTCGAAGTCC-3] served as replication control. Both thermal cycling conditions for nested PCR were the same: 35 cycles of denaturation for 45 s at 94°C, annealing for 30 s at 55°C, and extension for 1 min at 72°C, and a final incubation for 10 min at 72°C. The amplified PCR products were examined by 1.5% agarose gel electrophoresis.

IgM and IgG anti-HEV ELISA

All serum samples were thawed at room temperature and tested with IgM and IgG anti-HEV ELISA kits (manufactured by Genelabs Diagnostics). The samples with an optical density less than the cutoff value (mean optical density for the three negative controls on each plate plus 0.4 for IgM，plus 0.5 for IgG) were considered as negative. Samples with an optical density greater than or equal to the cutoff value were tentatively considered reactive and then retested to confirm the result. The absorbance was determined at 450 nm. The ELISA was performed according to the protocols provided by the manufacturer.

Statistical analysis

The IgG and IgM anti-HEV tests were compared with the HEV RNA test by RT-PCR for concordance. Fisher's exact test and the chi-square test were used to compare the prevalence of anti-HEV among tested groups. A *p* value of less than 0.05 was considered significant.

RESULTS

The specificity of nested RT-PCR for HEV

Nested RT-PCR for HEV was performed on 20 HAV patients, 30 HBV patients, 30 HCV patients and 30 healthy persons. All the results were negative. Besides, their anti-HEV IgM detection results showed negative as well. Nested RT-PCR for HEV was able to amplify specifically without any cross reaction with other hepatitis virus.

The frequency of IgM and IgG anti-HEV detected by ELISA

One hundred and twenty six (126) serum samples collected from 25 patients with sporadic acute hepatitis E during treatment were detected for IgM and IgG anti-HEV by ELISA. Our study showed that cases with positive results for IgM alone were 6.3% (8/126), IgG alone were 25.4% (32/126), and both for IgM and IgG were 56.3% (71/126). The frequency of HEV-negative cases both for IgM and IgG were 11.9% (15/126). In all, the frequency of HEV-positive for IgM was 62.7% (79/126), IgG 81.7%

Table 1. The nested RT-PCR results of 126 serial serum specimens of HE.

Anti-HEV	Number	Positive cases for nested PCR	Positive rate (%)
IgM+IgG-	8	2	25.00
IgM+IgG+	71	52	73.20
IgM-IgG+	32	10	31.30
IgM-IgG-	15	3	20.00
Total	126	67	53.20

Table 2. The test result of two methods comparing 126 serial serum specimens of HE.

HEV RNA result	anti-HEV IgM		Total
	Positive cases	Negative cases	
Positive cases	61	6	67
Negative cases	18	41	59
Total	79	47	126

X^2 = 49.159, P=0.000; P=0.023

(103/126).

Nested RT-PCR detection

HEV RNA was detected in 53.2% (67/126) of patients with sporadic acute hepatitis E, 25.0% (2/8) in anti-HEV-IgM-positive alone samples, 31.3% (10/32) in anti -HEV-IgG-positive alone samples, 73.2% (52/71) in anti-HEV positive both for IgM and IgG samples, and 20.0% (3/15) in anti-HEV negative for IgM and IgG samples(Table 1).

The agreement between IgM and nested RT-PCR for positive results was 77.2% (61/79), and the agreement for negative cases was 87.2% (41/47), with the total coincidence of 80.9% (102/126) (Table 2). Our study showed a concordance of the laboratory results for HEV between nested RT-PCR detection and IgM ELISA (*Kappa* =0. 613, *P*=0.000). Under analysis of *Pearson* X^2 and *McNemar* X^2, though with some complementarity, it was suggested that there was a significant difference between nested RT-PCR detection and IgM ELISA (X^2 = 49.159, *P*=0.000; *P*=0.023), so that they could not be replaced by each other for HEV detection.

Correlation between course disease and lab results for both assays

Among the 126 serum samples of 25 patients, 3 cases with positive results for HEV RNA showed negative anti-HEV IgM at first, and presented positive IgM in succession after the trail of detection. HEV RNA was detected in serum samples from sporadic acute hepatitis patient usually by day 1 to 12 after the onset of symptoms, but showed a decreasing sensitivity with the progression of disease. While anti-HEV IgM presented a high positive frequency up to week 2 after the onset of symptoms (Table 3).

DISCUSSION

HEV infection often causes viremia. Patients were found to excrete HEV in stool after there was a high concentration of virus in bile. Hepatitis E viremia can be detected before the onset of liver abnormality which was accompanied by humoral immune reaction. Currently, the exact moment at which HEV caused the antibody reaction was unknown. Besides, since there is no robust system to grow HEV in culture, there are some limitations in the field of HEV study (Chandra et al., 2008). Studies on experimentally infected macaques first defined the clinical and serological course of HEV infection. In those studies, serum anti-HEV immunoglobulin G (IgG) appeared around 3 to 4 weeks post-inoculation at the peak of ALT elevation. A human volunteer study showed anti-HEV IgM to peak in the symptomatic period and then decline to baseline within 3to 6 months of illness. Serum anti-HEV IgG levels continued to rise during the symptomatic phase and became detectable in the convalescent phase for 2 years (Meng, 2010; Zhu et al., 2008). Therefore, both molecular and serological methods are important for HEV diagnosis.

The diagnosis of hepatitis E is usually made serologically by commercial ELISA kits. However, limitations for serodiagnosis exist due to various envelope antigens in different kits. Since different HEV genotypes determine the differences of their antibody response to corresponding antigens, the detection results from different ELISA kit might present discordance (Chen et al., 2005). Evidences showed that anti-HEV antibodies, developed

Table 3. The result of 126 serial serum specimens detected by two methods of HE.

Course of disease (days)	Number	HEV RNA		anti-HEV IgM	
		Positive cases	(%)	Positive cases	(%)
1-4	24	16	66.7	14	58.3
5-8	45	28	62.2	30	66.7
9-12	21	12	57.1	15	71.4
13-15	17	7	41.2	11	64.7
>15	19	4	21.1	9	47.4
Total	126	67	53.2	79	62.7

in various laboratories using synthetic peptides or recombinant proteins derived from different genotypes and/or subtypes of HEV, showed a wide variation in sensitivity in seroprevalence studies (Zaki et al., 2009; Ma et al., 2011). Furthermore, derived from the same genotype and/or subtype of HEV but expressed in different expression system, the antigenicity and epitope displayed significance difference (Chau et al., 2006). Depending only on serology for hepatitis E can either miss the diagnosis of early cases or over-diagnose cases without true viremia. Thus aetiology will be of great help for HEV diagnosis. Methods for aetiological diagnosis include immune electron microscopy and traditional RT-PCR assay. These methods, with lower sensitivity, were limited to detect latent infection and early acute infection. Recently, nested RT-PCR shows a better way for detection with a higher specificity and sensitivity (Kumar et al., 2011; Mirazo et al., 2011).

HEV RNA was usually detected in both serum and stool in late latent period and early acute infection of patients with HE. In some HE cases with both negative results of anti-HEV IgM and IgG, HEV RNA was positive. In the present study, 3 of 15 serum samples with both negative results of anti-HEV IgM and IgG had detectable positive HEV RNA. It is believed that HEV RNA detection is of great help to make a diagnosis in HEV early infection, covering up the shortage of serological method. Since only genotype I and IV HEV were reported in China, we focused on genomic sequences of genotypes I and IV HEV and selected the conserved region for designing a set of nested RT-PCR primers, of which GC content was equal to AT content. We found that the selected sequence, located in the junction region between N-terminal sequence of ORF2 and ORF3 in HEV, showed a high conservatism, but were rich in GC that was not beneficial to PCR amplification. Sequences located in inner ORF2 was not only relatively conservative, but also had the appropriate GC content for PCR amplification (Ahmad et al., 2011; Johne et al., 2010). We thus selected a set of nested RT-PCR primers for this region and resulted in an expected high degree of specificity. HEV RNA was detected in 53.2% of patients with sporadic acute hepatitis E, which bled in 12 days after the onset of disease. Consistent with other relevant reports, the positive frequency of HEV RNA was lower than ELISA with 62.7 (anti-HEV IgM) and 81.7% (anti-HEV IgG), respectively. We assumed that it might be due to the short period of viremia, which usually lasted for only 2 weeks, and the presence of anti-HEV IgM often lasted longer than viremia (Cheng et al., 2012; Khudyakov and Kamili, 2011). In our study, the total coincidence of agreement between IgM and nested RT-PCR detection was 80.9% (*Kappa* = 0.613, *P* = 0.000) in 126 patients with sporadic acute hepatitis E. Under analysis of Pearson X^2 and McNemar X^2, though with complementarity, it was suggested that there was a significant difference between nested RT-PCR detection and IgM ELISA (X^2=49.159, *P*=0.000; *P*=0.023), so both molecular and serological methods must be applied for accurate diagnosis.

Noted above, HEV RNA was usually detected in early acute infection of patients with HE, and tended to decline to an undetectable level with the course of disease progressing. On the other hand, since anti-HEV IgM might not reach the detectable level in early infection stage, depending mainly on serological tests could mis-diagnose cases with early viremia before seroconversion. Instead, positive result of HEV RNA would be more helpful for early diagnosis. From this study, we could conclude that HEV RNA detection is of great clinical significance, which shows an obvious advantage of making a diagnosis in early infection of HEV.

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Full Length Research Paper

Induction of non protein thiols by chromium in cyanobacteria isolated from polluted areas

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Two cyanobacterial strains *Synechocystis* "AHZ-HB-MK" and "AHZ-HB-P2A" were isolated from tannery effluent form Lahore, Pakistan and their tolerance against K_2CrO_4 was examined. Although growth was completely inhibited at 10 µg mL^{-1} in AHZ-HB-P2A strain, cell growth of AHZ-HB-MK strain was observed even at a concentration of 100 µg mL^{-1}. To show reasons for different tolerance between the two strains, we compared changes in nonprotein thiols, glutathione (GSH and GSSG), cysteine and cystine, after K_2CrO_4 treatment in both strains. After K_2CrO_4 treatment GSH completely disappeared in both strains, suggesting that GSH was involved in detoxification. GSSG content in former strain remarkably increased almost three and six times after 7-days treatment with 10 and 100 µg mL^{-1} K_2CrO_4, respectively while it decreased in the later strain. Cysteine content in the former strain increased almost three and eleven times after 1 day and 7 days treatment with 100 µg mL^{-1} K_2CrO_4, respectively, such increase in cysteine content was not observed in later strain. These data revealed that former strain could induce glutathione and cysteine but later strain could not. The difference in the ability to induce the nonprotein thiols between two strains must be responsible for differential tolerance in K_2CrO_4 observed in the growth inhibition.

Key words: Chromium, cysteine, cyanobacteria, glutathione, nonprotein thiol, *Synechocystis*.

INTRODUCTION

Chromium is an essential trace element for organism (Anderson 1998; DiBona et al., 2011; Ghosh et al., 2002; Vincent, 2000) however, high concentrations of chromium are toxic (Cheng and Dixon,1998; Kim et al., 2002; Medeiros et al., 2003) carcinogenic (Singh et al., 1998) genotoxic (Godet et al., 1996) and teratogenic (Asmatullah and Shakoori, 1998). Chromium is used in the manufacture of alloys, corrosion inhibitory paints, wood preservatives, mordants and fixatives for dyes and tanning, photographic sensitizers and pigment for rubber and ceramics and as an anticorrosive in cooking systems and boilers (Cotman et al., 2004) and chromium pollution in aquatic environments is now threatening ecological systems and human health in several regions in the world (Leghouchi et al., 2009; Shankar 2009; Tziritis et al., 2012; Yang and Liu 2012).

Bioremediation is one of expected method to remove toxic chemicals from polluted areas. Autotrophic organisms such as plants and algae suit for bioremediation of inorganic chemicals, the process is known as "phytoremediation" (Ghosh and Singh, 2005; Jadia and Fulekar, 2009; Kumar and Goyal, 2009). Since cyanobacteria are

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Abbreviations: GSH, Glutathione reduced form; **GSSG,** glutathione oxidized form.

ubiquitous and their physiological and genomic backgrounds are well known as compared to other algae, hence they may be potential candidates for phytoremediation. In the present study, we isolated two strains of *Synechocystis* species from chromium polluted areas in Pakistan, and examined tolerance against chromium and changes of nonprotein thiols after chromium treatments.

Glutathione, the tripeptide γ-glutamylcysteinylglycine, is a major source of nonprotein thiols in most organisms. The major roles of glutathione are to protect cells from environmental stress such as oxidation which results in the production of reactive oxygen species, and from xenobiotic electrophiles and heavy-metal ions having high electronegativity by combining with the sulfhydryl groups (Meister, 1975; Meister and Anderson, 1983; Marrs 1996; Coleman et al., 1997). There is possibility of glutathione involvement in Zn, Ni and Cr uptake (Gharieb and Gadd, 2004). Cysteine is also a major source of nonprotein thiol, but its roles in detoxification and antioxidant activities have been paid less attention. However, since cysteine is usually more abundant than glutathione in higher plants (Duke, 1992), it is known that addition of cysteine together with chromium alleviate the toxicity of chromium in a cyanobacterium *Anabaena doliolum* (Duke, 1992). Thus, cysteine must act as the first line of defense against chromium. The aim of this study was to determine whether glutathione and cysteine are involved in tolerance against chromium in the isolated strains. Understanding of the mechanism of their tolerance is expected to give useful information on how to utilize them for phytoremediation of chromium.

MATERIALS AND METHODS

Plant material and growth conditions

Two strains of *Synechocystis* sp. were isolated from chromium-polluted effluents in the North Northeast of Pakistan. Strain AHZ-HB-P2A is from tannery effluent of Kasur district and strain AHZ-HB-MK is from tannery effluent in Muredkey locality near Lahore (Hameed and Hasnain, 2005) and their molecular identification was also performed (Hameed and Hasnain, 2012). The chromium concentration was 0.50 µg mL^{-1} in "AHZ-HB-P2A" strain isolation site (it was isolated from a pond of treatment plant) and 2.20 mg g^{-1} in AHZ-HB-MK strain isolation site (as it was isolated from moist soil of polluted site). The cells of both strains are solitary and mostly in pairs, have no mucilage or very fine narrow colorless sheath, cells are pale green in color and diameter is about 3 µm. The fluorescent tube lights used were of light intensity 5.8 to 7.8 µE m^{-2} s^{-1}. Cells were grown under a 12 h:12 h light-dark cycle at 28°C in BG11 medium (Rippka et al., 1979). Chromium was supplemented as K_2CrO_4 under same growth conditions for controls.

Extraction of nonprotein thiols

Procedure of extraction of nonprotein thiols was carried out following Satoh et al. (2002) with modification in which GSH was retained in its reduced form as per Anderson (1985). Nonprotein thiols were extracted at 6 h after starting the light period in the culture. Cells were collected from 300 mL of culture by centrifugation at 940 x*g* for 10 min and suspended in glass distilled water. Trichloroacetic acid was added to the cell suspension to final concentrations of 5%. The cells were completely disrupted by sonication with a Bioruptor (COSMO BIO, Tokyo, Japan) at 0°C and then separated by centrifugation at 11,000 x*g* for 15 min at 4°C. The supernatant was used for assays. In each extraction the number of cells in cultures was calculated from cell density measured using a hemacytometer.

Analysis of nonprotein thiols

Total GSH and GSSG were measured by the GSSG recycling method (Anderson, 1985) with GSSG as the standard. The final concentration of glutathione reductase (Boehringer Mannheim GmbH, Mannheim, Germany) was 0.5 unit mL^{-1}. Cysteine and cystine were measured using the acid ninhydrin technique of Gaitonde (1967). Nonprotein thiol recovery was determined by comparing GSH and GSSG contents between two samples from the same cell culture, one of which was externally added GSH prior to disruption of cells by sonication. Data were obtained from a minimum of three independent experiments. A *t*-test was used to determine statistical significance.

Estimation of cell biovolumes

Biovolumes of the strains AHZ-HB-MK and AHZ-HB-P2A were calculated to be 14.3 and 13.9 μm^3 respectively, according to the formulae of Hillebrand et al. (1999) considering the cell shapes of both strains to be sphere or spheroid. More than fifty cells were estimated from each strain. Cellular concentrations of non-protein thiols were calculated by dividing the non-protein content per cell by biovolume.

RESULTS

Figure 1 shows inhibition of growth by K_2CrO_4 in both *Synechocystis* strains AHZ-HB-MK and AHZ-HB-P2A. Cell of AHZ-HB-MK strain could grow under the conditions at 10 and 100 µg mL^{-1} of K_2CrO_4 although cell densities after 7 days were 1/14 and 1/23 as compared to the culture without K_2CrO_4. However, cell growth of AHZ-HB-P2A strain almost completely inhibited by 10 µg mL^{-1} K_2CrO_4, and even cell density slightly decreased after two days at 100 µg mL^{-1}. Interestingly, cell density of both strains slightly decreased after one day but increased after two day in the condition of 100 µg mL^{-1} K_2CrO_4, but the strain AHZ-HB-P2A could not grow the following days while the AHZ-HB-MK strain continued to grow. These results determine that AHZ-HB-MK strain is much more tolerant to K_2CrO_4 than AHZ-HB-P2A strain.

We examined changes in nonprotein thiols after K_2CrO_4 treatment in the two strains (Figures 2 and 3). Nonprotein thiols per intact cell of AHZ-HB-MK strain are 6.15 amol (corresponding to 0.43 mM) for GSH 10.7 amol (0.75 mM) for GSSG 17.3 amol (1.2 mM) for cysteine and 40.2 amol (2.8 mM) for cystine. Those for AHZ-HB-P2A strain are 6.74 amol (0.48 mM) for GSH and 6.59 amol (0.47 mM) for GSSG, 24.4 amol (1.76 mM) for cysteine and 13.0 amol (0.94 mM) for cystine. Content and concentration of each molecule is similar between the two strains

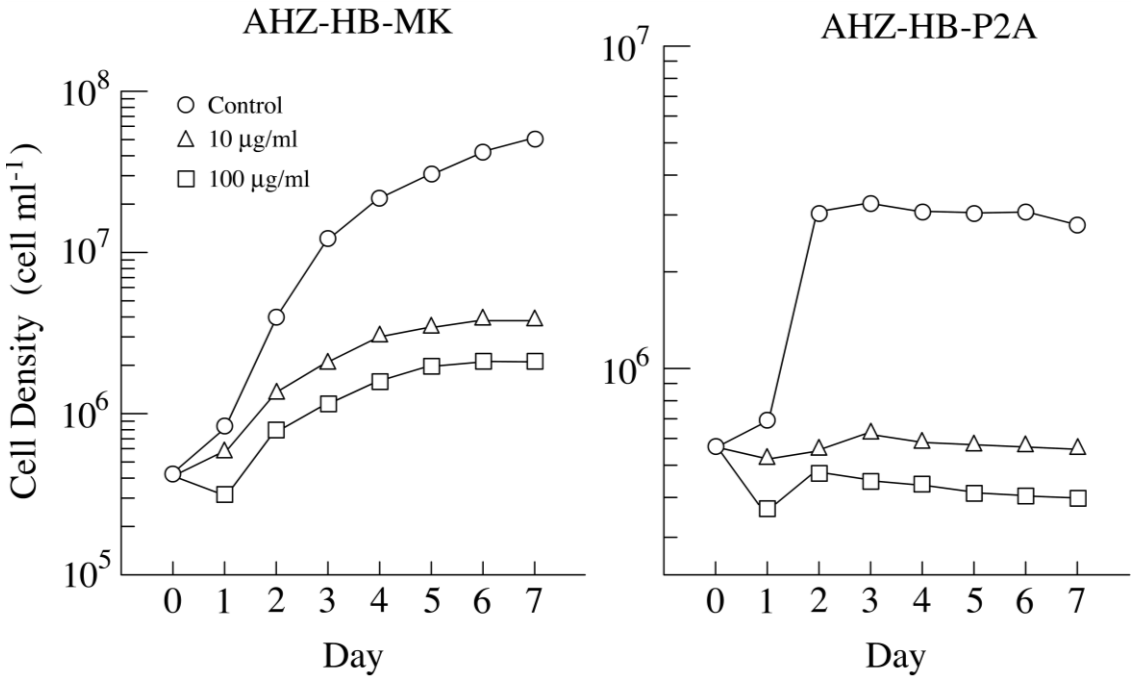

Figure 1. Growth inhibition by K_2CrO_4 in two strains of *Synechocystis* sp. AHZ-HB-MK and AHZ-HB-P2A. Cell density was measured using a hemacytometer after inoculating the cells from a full grown culture into medium containing 0, 10 and 100 µg mL^{-1} of K_2CrO_4.

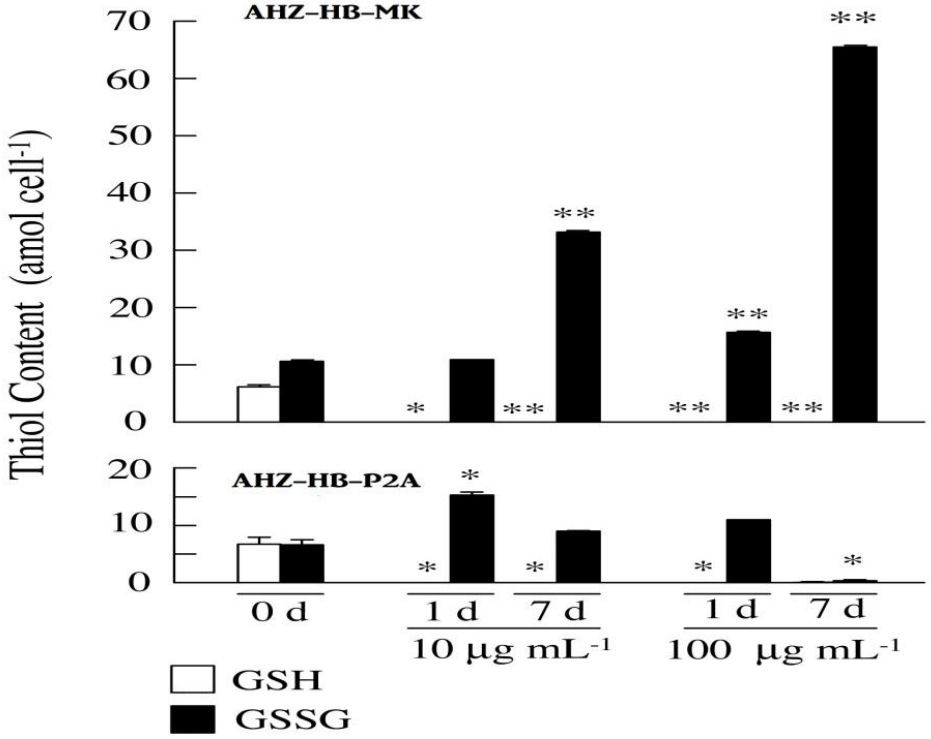

Figure 2. Changes in nonprotein thiol (Glutathione) content per cell after treatments with K_2CrO_4 in strains AHZ-HB-MK and AHZ-HB-P2A. Nonprotein thiols were extracted 0, 1 and 7 day after treatments with 10 and 100 µg mL^{-1} of K_2CrO_4. Data are means ± standard deviation from a minimum of three independent experiments. Significant differences between control (0 d) and chromium-treated one are indicated with asterisks (*t*-test; *$P < 0.01$; **$P < 0.001$).

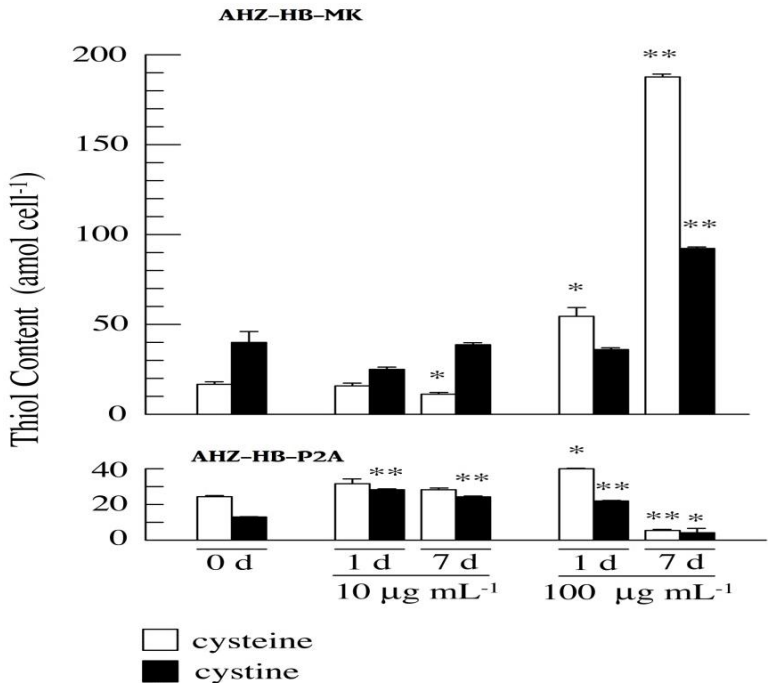

Figure 3. Changes in nonprotein thiol (cysteine and cystine) content per cell after treatments with K_2CrO_4 in strains AHZ-HB-MK and AHZ-HB-P2A. Cysteine and cystine were extracted 0, 1 and 7 day after treatments with 10 and 100 µg mL^{-1} of K_2CrO_4. Data are means ± standard deviation from a minimum of three independent experiments. Significant differences between control (0 day) and chromium-treated one are indicated with asterisks (*t*-test; **P* < 0.01; ***P* < 0.001).

except for cystine. Cysteine content in AHZ-HB-MK strain increased to almost three and 11 times after 1 and 7 days treatment with 100 µg mL^{-1} K_2CrO_4, respectively, whereas at 10 µg mL^{-1} some decrease or no change in its level was observed. Large increase in cysteine content was not observed in AHZ-HB-P2A strain; instead it remarkably decreased after 7 days at the concentration of 100 µg mL^{-1}.

DISCUSSION

Nonprotein thiols such as glutathione and cysteine are involved in detoxification against heavy metals, chromium (VI) for example, forms thiolate complex with reduced form of glutathione (GSH) and cysteine (Brauer et al., 1996; Suzuki, 1990; Wiegand et al., 1985). Furthermore, addition of cysteine together with chromium alleviates the toxicity of chromium in a cyanobacterium *Anabaena doliolum* (Duke, 1992). After K_2CrO_4 treatment GSH completely disappeared under all conditions in both strains, suggesting that GSH was involved in detoxification. GSSG content in AHZ-HB-MK strain remarkably increased to about three and six times after 7-days treatment with 10 and 100 µg mL^{-1} K_2CrO_4, respectively. However, the change in GSSG content in AHZ-HB-P2A strain showed a different pattern from AHZ-HB-MK strain. GSSG content of AHZ-HB-P2A strain slightly increased after 1 day at the both concentrations, but it decreased after 7 days, especially at the concentration of 100 µg mL^{-1}.

The data revealed that AHZ-HB-MK strain could induce glutathione and cysteine but P2A strain could not. Difference in the ability to induce nonprotein thiols between the two strains might be responsible for the difference of the tolerance against K_2CrO_4 observed by the inhibition of growth. Although GSH was completely depleted after K_2CrO_4 treatment but cysteine was not, rather the ratio of cysteine/cystine became higher after their induction by 100 µg mL^{-1} K_2CrO_4 in AHZ-HB-MK strain. It has also been reported that time-dependent decrease of reduced glutathione (GSH) with an increase of oxidized glutathione (GSSG) level suggested GSH was involved in detoxification of MC-LR in the liver (*Jinlin* et al., *2011*). There are possibilities of activation of cysteine reductase, the synthesis of new cysteine or both. It has also been reported that redox of cysteine and glutathione must be controlled independently in marine phytoplankton (Satoh et al., 2002).

In this study, we measured intracellular contents and concentration of nonprotein thiols in two strains of *Synechocystis* genus. Concentrations of glutathione in plants, algae, bacteria, and animal tissues range from 0.1 to 12 mM (Meister, 1975; Satoh et al., 2002; Coppellotti,

1989). Our results showed that the glutathione concentrations of the two strains of *Synechocystis* are within this range under the condition without chromium. Information about intracellular concentration of cysteine in organisms is limited, but it is known that it ranges from 0.6 to 12 mM in several species of eukaryotic algae (Satoh et al., 2002; Coppellotti, 1989). Cysteine concentrations of the two strains of *Synechocystis* are also within this range. Although concentrations of nonprotein thiols are similar before chromium treatment in two strains, AHZ-HB-MK and P2A, the concentrations become very different after chromium treatment, with large increase in AHZ-HB-MK strain and decrease in AHZ-HB-P2A strain. It indicates that glutathione and cysteine are involved in detoxification of K_2CrO_4 and that the control mechanism of levels of glutathione and cysteine are more sensitive in AHZ-HB-MK strain than that in AHZ-HB-P2A strain. Involvement of detoxification mechanisms other than glutathione and cysteine against K_2CrO_4 related to the difference of the tolerance is uncertain. Cyanobacteria are known to have a cysteine-rich metal-binding protein, zinc metallothionein (SmtA), in response to elevated concentrations of zinc, cadmium and copper (Cavet et al., 2003). Mutants of *Synechococcus* PCC7942 lacking *smtA* showed reduced tolerance to elevated zinc levels (Turner et al., 1993), and conversely, mutants over-expressing SmtA showed increased zinc tolerance (Turner et al., 1995). However, no evidence has been reported that SmtA is induced by chromium nor involved in chromium detoxification. Furthermore, since the two strains shows the same tolerance against $ZnSO_4$ (data not shown) SmtA must have no relation with the different tolerance level against K_2CrO_4. AHZ-HB-MK strain will be a candidate for bioremediation of chromium pollution if cells are found to accumulate chromium in a high level or if they modify chromium to less toxic compounds, from chromium (VI) to chromium (III) for example, even if they release chromium to the environment.

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Full Length Research Paper

Antimicrobial screening of crude extracts from the indigenous *Ganoderma lucidum* mushrooms in Namibia

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The indigenous *Ganoderma* mushroom belongs to the class Basidiomycetous. It has been used in different systems of traditional medication for the treatment of diseases of human beings and animals. It contains various triterpenes, polysaccharides, alkaloids and steroids known to have broad effects pharmacologically and anti-bacterial properties. The indigenous *Ganoderma* mushroom has been used by locals in Namibia traditionally as a source of medicine to fight skin and wound infections, and other ailments. There is a need to validate the traditional usage of *Ganoderma* mushroom extracts on *Escherichia coli, Alcaligenes faecalis, Proteus vulgaris, Neisseria meningitidis, Bacillus cereus* and *Staphylococcus aureus*. This is important for the Namibian local communities, where people do not have access to modern medicines and use traditional medicines. This study analyzed the anti-bacterial effects of crude organic and aqueous extracts of the mycochemical components of the indigenous *Ganoderma* mushroom. Using the agar disc diffusion method, the crude extracts of the *Ganoderma* mushroom exhibited various degrees of inhibition against the tested organisms. The widest inhibitory zones (19.0 mm) were obtained with the crude benzene extract of *G. lucidum* against *E. coli* and *N. meningitidis.* The lowest zone of inhibition (6.0 mm) was demonstrated with the aqueous extract against *E. coli.* The study has concluded that the crude extracts of the indigenous Namibian *Ganoderma* mushroom possess antibacterial properties to all Gram positive and negative strains tested.

Key words: *Ganoderma lucidum*, antibacterial properties, *Escherichia coli, Alcaligenes faecalis, Proteus vulgaris, Neisseria meningitidis, Bacillus cereus* and *Staphylococcus aureus*.

INTRODUCTION

In recent years, there have been a significant number of human pathogenic bacteria becoming resistant to anti-microbial drugs (Donadio et al., 2002) and this is in part due to the misuse and overuse of available antibiotics (Monroe and Polk, 2000). Antimicrobial drug resistance is of major economic concern and impacts on physicians, patients, health care administrators, pharmaceutical pro-ducers and the public (McGowan, 2001). In addition, bacterial and fungal pathogens have complicated the treatment of infectious diseases (Baratta et al., 1998). Given the increase in multiple drug resistance of human pathogenic microorganisms, it is imperative that new and effective therapeutic agents be developed.

Traditional tribal communities, indigenous peoples and the eastern world have been using plants, spices and fungi for thousands of years as therapeutic agents. For the past decades, attention has turned to extracts and biologically active compounds used in traditional herbal medicine to uncover the scientific basis of their remedial effects and to seek new lead compounds for develop-ment into therapeutic drugs (Cragg et al., 1997). In addition to plants extracts as sources of antimicrobial

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Picture 1. *Ganoderma lucidum*.

agents, research is being performed on fungi for their ability to mobilize the body's humoral immunity and in turn prevent bacterial, viral, or fungal pathogens that are resistant to current therapeutic agents (Wasser and Weis, 1999c). Fungi are well known for the production of important antibiotic compounds such as penicillin, however, the occurrence of antibiotics in the class of fungi known as Basidiomycetes (the mushrooms) is scarcely documented (Miles and Chang, 1997) and there are only few reviews that summarizes the antibacterial activity from these organisms (Gao et al., 2003; Zjawiony, 2004). *Ganoderma* species belong to the Basidiomycetes class of mushroom, which is known to possess a variety of biochemical compounds with a wide range of pharmacological effects. *Ganoderma* species have been widely investigated for their therapeutic properties as antitumor and antiviral agents but have been far less investigated as a source of new antibacterial agents. A review by Gao et al. (2003), on the antibacterial and antiviral value of *Ganoderma* species supported this observation.

In Namibia, especially in the northern and northeastern part, *Ganoderma* have been used in relieving stress when sniffed as ash mixed with tobacco, calming of nerves when put in water, used as a drink. It is also used in the treating of cold and flu symptoms when its smoke is inhaled. Its extracts are applied to infected skin and to treat the wounds on children's heads. Kadhila-Muandingi (2010) and Ekandjo and Chimwamurombe (2012) reported the same traditional uses of *Ganoderma* mushroom in Ohangwena and Oshana regions of Namibia. Yongabi et al. (2004) also reported the use of crushed *Ganoderma* mushroom mixed with ash as ointment in the treatment of skin infections in Cameroon.

Locally, this mushroom is also said to have a history in treating animal diseases, especially cattle when suffering from lung diseases and goats with skin rushes (Kadhila-Muandingi, 2010; Yongabi et al., 2004).

There appear to be an increasing number of reports on Gram-positive bacteria developing resistance to virtually every clinically available drug (Donadio et al., 2002), and Basidiomycetous mushrooms have been shown to possess antibacterial activity against this group of bacteria. Other work showed that extracts were also active against Gram-negative organisms, *Proteus vulgaris* and *Escherichia coli*, *in vitro* (Yoon et al., 1994). Overall, extracts from mushrooms are observed to be more active against Gram-positive bacteria than Gram-negative bacteria (Smania et al., 1999).

It should be noted that the indigenous knowledge of medicinal mushroom use is linked to local culture and history (Opige et al., 2006). To our knowledge no studies have been conducted to validate the assessment of traditional medicinal uses of the indigenous *Ganoderma* mushrooms in Namibia. For this reason, this study was carried out to determine the antimicrobial properties of the aqueous and organic crude extracts from the fruiting bodies of the indigenous *Ganoderma* mushroom against *E. coli*, *Staphylococcus aureus*, *Bacillus cereus*, *Neisseria meningitides*, *Alcaligenes faecalis*, *Proteus vulgaris* which are known to cause wound infections, intestinal and urinary-genital tract infections, skin infections and abdominal cramps/diarrhea.

MATERIALS AND METHODS

Sample collection

Ganoderma lucidum (Picture 1) samples were collected from the natural environment of the northern and northeastern part of Namibia (namely Oshana, Ohangwena, Kavango and Caprivi regions). The mushroom fruiting bodies were identified by its shelf-like with a short stalk, and leathery to corky when fresh. They were present singly or in overlapping clusters at or near ground level. The upper surface is dark reddish brown and has a thin, shiny, varnish like crust that becomes coated with a layer of dull brown basidiospores. The pore surfaces are creamy white, becoming light buff and bruising dark brown, according to Van der Westhuizen and Eicker (1994).

Organic and aqueous extraction

The anti-bacterial compounds were extracted from *Ganoderma* mushroom samples with aqueous and organic solvents, in order to separate the chemical constituents into groups of different polarities. First, a "successive step extraction" was applied to determine the polarity of the mycochemical antibacterial compounds. Different solvents used present different polarity in order to extract successively compounds of different polarities: lipids, sterols, triterpenoids, glycoproteins, glycosides, sugars, amino acids and proteins. The solvents were used in order of increasing polarity: Benzene, chloroform, ethyl acetate (EA), ethanol (EtOH), methanol (MetOH) and aqueous extraction as hot water (HW) extract. All reagents were of analytical grade and were used as received.

About 45 g of each powdered sample was soaked and cold extracted with 400 mL of the organic solvents at room temperature for 5 days. The organic solvents were used successively with gradient polarity starting with benzene, chloroform, EA, EtOH, MetOH and aqueous extraction (HW). The crude extracts were gravity filtered through a 0.45 µm Whatman No. 2 filter paper. The filtrates were concentrated by evaporating excess solvent in a hot water bath and stored in the dark.

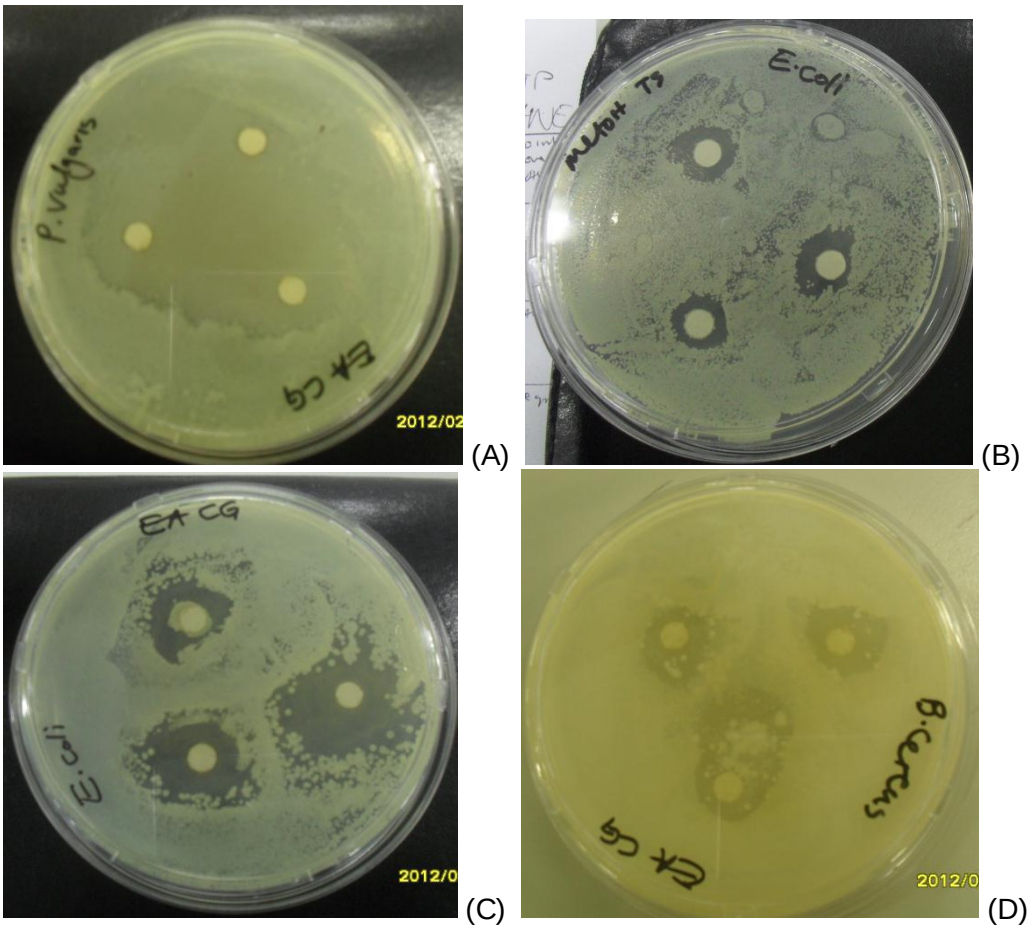

Figure 1. Positive zones of inhibition of (A) crude EA *Ganoderma* extracts against *P. vulgaris,* (B) crude methanol *Ganoderma* extracts against *E. coli*, (C) and (D) crude EA *Ganoderma* extracts against *E. coli* and *B. cereus*. Pure solvents were used as positive controls and no inhibition was observed.

Culturing of microorganisms and antibacterial testing

Lyophilized test microorganisms: *E. coli* (ATCC 25922), *S. aureus* (ATCC 25923), B. *cereus* (ATCC 10876), *N. meningitides* (Y) (ATCC 35561), A. *faecalis* (ATCC 8750), *P. vulgaris* (ATCC 33420) were obtained from the University of Namibia Microbiology Laboratory. They were grown on Nutrient Broth and further grown on Mueller-Hinton agar (MHA). All microorganisms were grown at 37°C. A disc-diffusion method was used to determine the antimicrobial activity of the crude *Ganoderma* mushroom extracts as described by Kisangau et al. (2007). Paper discs (6 mm, diameter) were dipped into the mushroom crude extracts using sterilized forceps and placed on the surface of the inoculated Petri dishes (Kisangau et al., 2007). The plates were incubated at 37°C for 24 h and observed for zones of inhibition. The anti-bacterial activity of each extracts was recorded by measuring any zone of growth inhibition diameter around the disc with a millimeter ruler. The experiments were done in triplicate and the average values were tabulated.

RESULTS

Antimicrobial sensitivity test

The tests revealed that the crude extracts of the indigenous *Ganoderma* mushroom of all different polarities as presented by the solvents used contain antimicrobial effects. The solvent used for each extraction was used as the negative control for each extract. The qualitative and quantitative analysis of each crude extract will be published once available in a separate publication.

DISCUSSION

Results from the tests indicate the presence of more than one mycochemical class of compounds of different polarities having antibacterial effects on the microbial strains tested. The inhibitory activities of all crude organic and aqueous extracts (Figure 1) were investigated against Gram-positive and Gram-negative bacteria. The results indicated that both aqueous and organic extracts from *Ganoderma* possessed activities against all tested microbial strains. The results presented in Table 1 show the inhibition zones of crude organic and aqueous extracts against all the microbial strain tested. Benzene crude extracts showed the largest inhibition zone as compared to other crude organic solvents against *E. coli*, *S. aureus*, *B. cereus*, *N. meningitidis*, *A. faecalis* and *P. vulgaris*.

Table 1. The disc diffusion assays inhibition zones of the crude organic extracts against the tested microbial strains. Pure solvents were tested as controls and showed no inhibition.

Test bacteria		Antibacterial activity of crude aqueous and organic extracts (mean values) (mL)					
		Benzene	Chloroform	EA	EtOH	MetOH	HWE
Gram +	*A. feacalis*	6.0s	6.0s	[8.0]	NT	6.0s	9.0
	B. cereus	10.0	8.0	7.0	NT	[7.0]	-
	E. coli	19.0	11.0	[8.0]	11.0	9.0	8.0
Gram -	*S. aureus*	[7.0]	10.0	6.0s	NT	10.0	10.0
	P. vulgaris	[8.0]	6.0	[7.0]	NT	12.0^{+}	[8.0]
	N. meningitides	19.0^{+}	14.0^{+}	[7.0]	NT	11.0	18.0

Zone of inhibition >5 mm was considered positive. S, a very slight zone of inhibition observed; [ ], incomplete inhibition was observed (reduced growth, but not complete elimination of growth); +, a double zone of inhibition was observed. The larger second zone, was a zone of incomplete inhibition, indicated by the parentheses [ ]; -, no inhibition was observed; NT, not tested; Pure solvents were tested as controls, they showed no inhibition (results not shown). Results shown are the average of triplicate discs and triplicate experiments performed on different days.

The maximum inhibition zone of the crude aqueous extract of the *G. lucidum* fruit bodies obtained was 18.0 mm against *N. meningitidis* and minimum of 8.0 mm against *E. coli* and *P. vulgaris.* Whereas the maximum inhibition zone of crude organic solvents extracts against the test microbial strains obtained was 19.0 mm against *E. coli* and *N. meningitidis* of benzene extracts, and a minimum of 6.0 mm of benzene extracts against *A. feacalis*, 6.0 mm of chloroform extracts against *P. vulgaris* and *A. feacalis*, 6.0 mm of EA extracts against *S. aureus*, and 6.0 mm of MetOH extracts against *A. feacalis*. Crude benzene, EA extracts appeared to have the strongest antibacterial effect therefore a larger inhibition zone. It could be that benzene extracted most of the non-polar components while EA extracted most of the polar components of the mushrooms thus exerted the most antimicrobial effects.

This is in agreement with activities observed for the benzene extracts against the tested microorganisms of the Gram-positive bacteria *B. cereus* (Roberts, 2004). Also in agreement with reports of Ofodile et al. (2005) that aqueous extracts of *Ganoderma* exhibited inhibitory activity towards the *Bacillus* species.

It showed be noted that the organic solvents used for extraction were assayed as negative controls, of which no inhibition zone activities were recorded and observed against the tested microbial strains.

The different antibacterial activities observed may be due to different mycochemical compounds which were detected in the fruiting bodies of the indigenous *Ganoderma* mushroom. *Ganoderma* is known to possess various chemical compounds such as triterpenoids, flavanoids, coumarins, quinones, carotenoids and amino acids as having antibacterial properties (Roberts, 2004).

There have been reports showing that triterpenes have a great antibacterial effect (Wilkens et al., 2002) and it is well documented that triterpenes are one of the major constituents isolated from *Ganoderma*.

S. aureus is an important causative agent of invasive skin diseases including superficial and deep follicular lesion (Usman et al., 2007). Therefore, antibacterial activity detected in the benzene crude extracts of the indigenous *Ganoderma* mushroom against *S. aureus and P. vulgaris* support the use of this indigenous *Ganoderma* mushroom to treat skin and wound infection as indicated by local people.

Conclusion

This study demonstrated that the indigenous *Ganoderma* mushrooms may have a good potential for the production of useful bioactive metabolites and they may serve as a good source for antimicrobial drugs. Effective concentrations of the active organic extracts were not investigated in this study and will be published once available in a separate publication. This study provides justification to conduct further research to evaluate and characterize the antibacterial activity of the indigenous *Ganoderma* mushroom extracts on a wider range of clinically relevant microbes.

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Full Length Research Paper

Medical importance of *Lactobacillus fermentum* lysate as a bioactive agent against some pathogenic *Candida* and *Aspergillus* strains

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In search of a safe and new biosource for treating fungal infections, the bacterial lysate of probiotic *Lactobacillus fermentum* was tested for its antifungal activity against *Aspergillus niger*, *Aspergillus flavus* and *Candida albicans*. The antifungal activity of lysate was analyzed by dry weight, disc diffusion and micro broth dilution methods. The growth of *A. niger* and *A. flavus* was inhibited by 31 µg lysate protein/disc but *C. albicans* was firstly inhibited at 62 µg lysate protein/disc. MIC for inhibition of *A. niger* was recorded as 125 µg lysate/ml while, *A. flavus and C. albicans* were inhibited at MIC of 62 µg lysate/ml by micro broth dilution assay. De Man Rogose and Sharpe medium supplemented with wheat bran, corn steep liquer and yeast extract showed the highest yield, 10 g dry biomass/L of *L.fermentum* at pH 6.8. The elution profile of the purified lysate showed five fractions, the first gave more than 60% of the original sample. Its MIC was 25 µg lysate/disc. Its molecular weight was 20 to 30 KDa. Toxicity tests revealed that, up to 150 µg lysate/ml showed no significant haemolysis.

Key words: Haemolysis, *Lactobacillus fermentum*, lysate, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

INTRODUCTION

The number of emerging or re-emerging bacterial, fungal and viral pathogens continues to increase. Additionally, multi drug resistance is also on the increase, in both nosocomial and community-acquired pathogens. The mortality rate due to invasive aspergillosis has risen steadily with a 357% increase which caused significant morbidity and mortality during 1980-1997 (Morgan et al., 2005; Samson et al., 2004). Thus, the need for new antimicrobial agents is perhaps greater now than it has ever been. In recent years, antibacterial and antifungal peptides have gained a lot of interest, due to their potential use as a new generation of therapeutic agents (Zasloff, 2002). Antifungal proteins such as ribosome-inactivating proteins (Ng and Parkash, 2002) and glucanases (York et al., 2004) have been reported from a variety of sources including bacteria, mammals, insects and plants for treating fungal infections. A novel anti-fungal pyrrole derivative was recorded from *Datura metel* leaves (Dabur et al., 2004, 2005). Screening of three novel antimicrobial peptides was assayed for their activity against crop fungal pathogens (Lan et al., 2011).

A protein isolated from *Penicillium* CL showed activity against 21 fungal isolates (Galgoczy et al., 2005). Pro-duction by *Bacillus pumilus*, of an antifungal compound that is active against Mucoraceae and *Aspergillus* species was reported by Bottone and Peluso (2003). A cytosolic

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protein (PPEBL21) was purified from *Escherichia coli* BL21 that demonstrated potent antifungal activity against pathogenic strains of *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger* and *Candida albicans* (Yadav et al., 2005a). Bacterial lysates are powerful inducers of a specific immune response against bacterial infections, but their mechanism of action is not fully understood. Apparently, they lower bacterial carriage levels in the respiratory tract, decrease hospitalizations owing to exacerbations, and reduce the need for antibiotics (Cazzola et al., 2012).

In view of the importance of probiotics in human health, the present study was undertaken to investigate the effect of cytosolic proteins from *L. fermentum* as a bioactive agent against some human pathogenic fungi.

MATERIALS AND METHODS

Microorganisms

L. fermentum used in the present study was isolated from commercially dairy products in the market and was identified at the center of El Azhar University, Cairo, Egypt according to Bergey's manual of determinative bacteriology, 1986 and 1994. De Man Rogose and Sharpe medium (MRS), is specific for the isolation, growth and enrichment of lactic acid bacteria (Samson et al., 2004). Fungal strains used as indicator organisms for the assessment of the antifungal activity were isolated from garden soil and they are: *A. niger, A. flavus. C. albicans* kindly obtained from Immunology and Microbiology Department, Faculty of Medicine, Alexandria University.

Preparation of the bacterial lysate (BL)

Three days exponential phase cells were separated by centrifugation at 12000 xg for 30 min. The pellets were suspended in 10-15 ml sonication buffer (50 mmol Tris/HCL, 50 mmol EDTA) adjusted to a pH 8. Cells suspension was then sonicated for 1 min/ml of suspension at 50% power/cooling periods using a soincator (Bandelin Sonopuls HD2070 Maxpower). The sonicated samples were centrifuged at 12000 xg for 20 min and the supernatants were used as crude lysate which were then freeze-dried (Yadav et al., 2005b). Protein concentrations of the bacterial lysates were determined by Lowry et al. (1951) before and after lyophilization.

Antimycotic activity determination

Disc diffusion method

Sterilized filter discs were impregnated with different concentrations (31 – 500 µg/ml) of the bacterial lysate and were placed on the surface of agar plates already inoculated with 100 µl of fungal spore suspension. These plates were kept overnight in a refrigerator to allow lysate diffusion, and were then incubated at 37°C for 24 and 48 h. At the end of incubation period, the radial growth and zones of inhibitions were measured in mm. For negative control, boiling inactivation of bacterial lysate of *L. fermentum* was used. The previous procedures were repeated at least three times, mean and standard deviation were calculated.

Micro broth dilution

The test was performed in sterile non-coated 96-well culture plates. Autoclaved glucose peptone broth (90 µl) was added to each well of culture plates. Various concentrations of bacterial lysates (31.9 - 1000.0 µg/ml) were prepared in the wells and inoculated with 10 µl of spore suspension containing approximately 1 x 10^6 spores. The plates were incubated at 37°C and examined macroscopically after 48 h for the growth of the tested strains. MIC was determined.

Dry weight method

Flasks containing 30 ml of glucose peptone medium were inoculated with 100 µl of spore suspension of the provided fungal strain. Bacterial lysate under test was added after filter sterilization at different concentrations. One set of flasks was incubated for 24 h and the other for 48 h, under shacked conditions (150 rpm) at 32 ± 2°C. Hyphal growth of the tested fungus was collected by filtration, washed with sterile physiological solution, allowed to dry at 60°C for 24 h. The fungal dry mass was determined gravimetrically. By measuring the percentage of reduction in biomass of the tested fungal strains using the gravimetrical method (Rybka and Kailasaparty, 1996), these assays were repeated at least 3 times.

Optimization of a bioactive bacterial lysate production from *L. fermentum*

Selection of the most suitable carbon source

Carbon source which was found in MRS medium (glucose) was substituted with molasses, wheat bran, whey, sucrose, manitol and lactose each at a time. Each carbon substrate was used as 2% (wt/vol) and all other medium components left unchanged.

Selection of the most suitable nitrogen source

Yeast extract, beef extract and peptone of the MRS medium was substituted with yeast extract only, yeast extract with CSL (corn steep liquor), CSL only, NH_4Cl with CSL, and peptone each at a time. Each nitrogen source was used as 1% (wt/vol) and all other medium components left unchanged.

Fractionation of L. fermentum lysate

The lysate prepared from the *L. fermentum* was fractified by two methods. (1) Ultra filtration using Vivaspin® dividing it into 2 parts according to its molecular weight (KDa). (2) Fractionation by ion-exchange chromatography, DEAE-Cellulose (Patterson et al., 1980). A 15.0 mg of lysate protein was dissolved in Tris/HCl, pH 7.4, and loaded onto a DEAE-cellulose column pre-equilibrated with the same buffer. The non-adsorbed proteins were eluted with Tris/HCl, pH 7.4. The elution of adsorbed proteins was carried out using a 200 ml linear gradient from 0 to 1 mol of NaCl in Tris/HCl, pH 7.4. The flow rate was adjusted to 1.0 ml min^{-1} and 2.0 ml fractions were collected. The OD_{280} (optical density) of the fractions was measured. The OD_{280} values of various fractions were plotted against the human fractions numbers. The peaks were pooled and analyzed for antifungal activity. The proteins recovered in peaks showing antimycotic activity were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli and Favre, (1973).

Table 1. Antifungal bioactivity of lysate from *L. fermentum* against *A. niger*, *A. flavus* and *C. albicans* investigated by measuring the diameter of inhibition zone (disc diffusion assay).

L. fermentum (µg protein lysate/disc)	Diameter of inhibition zone (mm)		
	A. niger	*A. flavus*	*C. albicans*
31	1.8 ± 0.2	1.5 ± 0.1	0.0 ± 0.0
62	2.0 ± 0.2	2.0 ± 0.1	1.5 ± 0.1
125	3.2 ± 0.3	3.3 ± 0.4	2.5 ± 0.2
250	3.6 ± 0.4	3.7 ± 0.2	2.9 ± 0.1
350	3.9 ± 0.2	3.8 ± 0.2	3.1 ± 0.1
500	4.0 ± 0.3	4.2 ± 0.3	3.8 ± 0.2

Table 2. Antifungal bioactivity of lysate from *L. fermentum* against *A. niger*, *A. flavus* and *C. albicans* investigated by Microbroth-Dilution Assay.

L. fermentum (µg protein lysate/ml)	Microbroth-dilution assay		
	A. niger	*A. flavus*	*C. albicans*
1000.00	+	+	+
500.00	+	+	+
250.00	+	+	+
125.00	+	+	+
62.25	−	+	+
31.25	−	−	−

+, Complete inhibition; −, growth was observed.

Haemolytic assay

Erythrocytes were collected from apparently healthy individuals under physician supervision according to the basic method of Latoud et al. (1986). Erythrocytes were then separated by centrifugation. The pellets remained were washed three times with PBS and centrifugation at 1500 xg for 10 min. Different concentrations of bacterial lysates were prepared and 2% erythrocyte suspension was added to each concentration. The mixtures were incubated at 37°C for 1 h. After incubation period, erythrocytes were pelleted at 5000 xg for 10 min. The supernatant was collected and the absorbency at 450 nm was determined using a spectrophotometer. In negative control sets, only buffer was used for background lysis, whereas in positive controls, lysis buffer was used for completely lysing the erythrocytes.

RESULTS

Antifungal activity of the bacterial lysate

The antifungal activity of the prepared lysate from *L. fermentum* was examined by determination of growth inhibition firstly by measuring the diameter of inhibition zone by disc diffusion assay method. Table 1 shows that *L. fermentum* lysate exhibited a mild to moderate activity against *A. niger, A. flavus* and *C. albicans*. 31 µg protein lysate/disc inhibited the growth of *A. niger, A. flavus* but did not show any effect on *C. albicans* using the Disc-Diffusion Assay. One fold increase (62 µg protein lysate/ disc) of lysate concentration, leads to an increase of inhibition reaching 10 and 25% when using *A. niger* and *A. flavus* as test strains. However, an inhibition zone of 1.5 mm was first observed using *C. albicans* at the same concentration of lysate.

Determination of the minimum inhibitory concentration of lysate (MIC) as well as examining the morphological characteristics of the tested lysate-treated fungal strains was carried out using the Microbroth-Dilution Assay. Table 2 showed that for inhibiting the growth of *A. niger,* 4 folds of *L. fermentum* lysate concentration were required and the MIC was 125 µg lysate/ml. On the other hand, *A. flavus and C. albicans* were inhibited at MIC of 62.3 µg lysate/ml. The morphological appearance of the tested strains was observed microscopically from the microdilution wells as shown in Figure 1. *A. niger* showed normal appearance of heads however, the lysate treated spores showed atrophied heads and spores rarely germinated. *A. flavus* on the other hand, was grown normally in untreated spores and no growth was observed in case of lysate treated spores. *C. albicans*, unicellular fungal cells grown individually in lysate untreated cells, nevertheless, lysate treated cells showed frequent formation of pseudomycelia in which daughter cells did not separate from the mother cells. Fungal spores which were treated with Exoderil drug showed collapsed spores mass in the case of *A. niger* and *A. flavus* but *C. albicans* showed no pseudomycelium formation, but cells appeared empty or with abnormal vacuolation.

The percentage of reduction in biomass of the tested fungal strains was determined by gravimetrical method in which spores of *A. niger, A. flavus* and *C. albicans* were allowed to grow in lysate free-glucose peptone medium as a control test and in glucose peptone medium supplemented with 40 to 200 µg lysate/ml. A linear trend of inhibition was observed in all strains, however, the slope was higher in the case of *C. albicans* indicating strong inhibition effect, followed by *A. flavus* and then *A. niger* which showed the lowest slope but an inhibition percenttage in biomass of 65 as compared to spores grown in glucose peptone lysate free medium as shown in Figure 2.

Effect of carbon source on the growth and antifungal activity of *L. fermentum*

As shown in Table 3, MRS medium supplemented with wheat bran showed the highest productivity reaching 10 g dry biomass/L. The antifungal activity of lysate derived from *L. fermentum* cells grown on molasses showed the highest antifungal activity, which resulted in 2.1, 2.3 and 2.8 mm inhibition zones by using 300 µg lysate/ml when it was tested against *A. niger, A. flavus* and *C. albicans*, res-

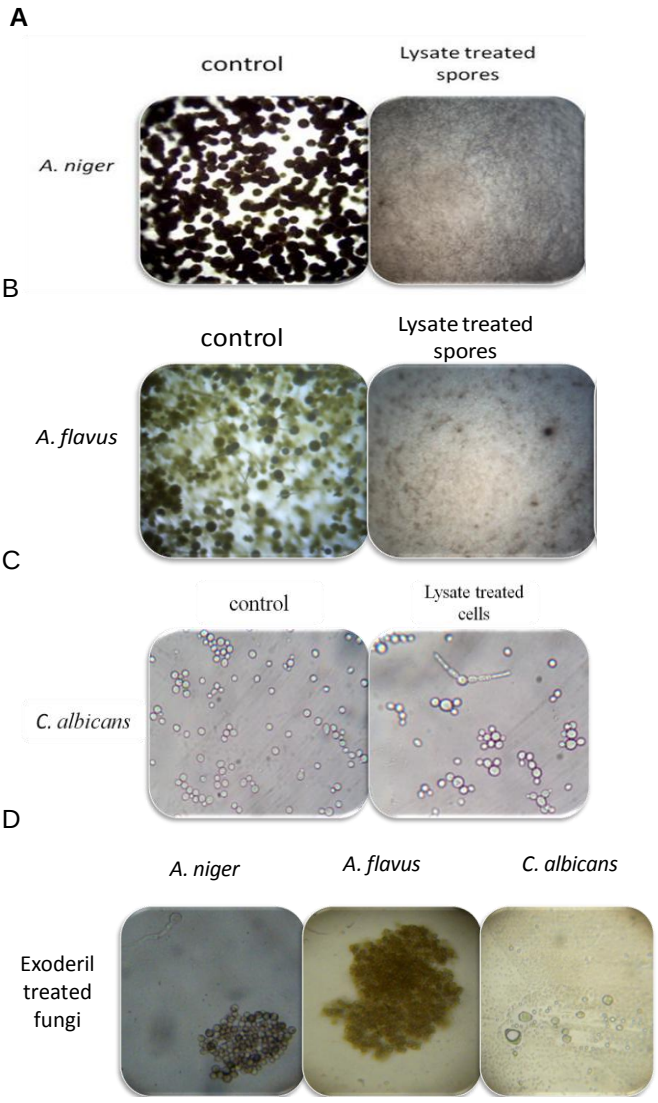

Figure 1. Micrographs of microwells showing growth of: A, *A. niger* spores without the addition of lysate (control) and lysate-treated spores with 125 µg ml^{-1} lysate. B and C, *A. flavus* and *C. albicans* spores without the addition of lysate (control) and lysate-treated spores with 62 µg ml^{-1} lysate. D, growth of *A. niger, A. flavus* and *C. albicans* in medium containing 20 µg ml^{-1}Exoderil. The microwells plates were incubated at 28°C for 48 h, examined microscopically and micrographs were taken.

pectively. The percentage of reduction in biomass of the tested fungal strains was determined. Spores were allowed to grow in lysate free-glucose peptone medium as a control test and in glucose peptone medium supplemented with 200 and 300 µg lysate/ml. Figure 3a, b and c showed that MRS medium supplemented with molasses provided a high and significant inhibition against *A. niger* and *A. flavus*, while MRS medium supplemented with wheat bran showed slight inhibition against *C. albicans.*

Effect of nitrogen source on the growth and antifungal activity of *L. fermentum*

Results illustrated in Table 4 indicated that lysate prepared from MRS supplemented with yeast extract and CSL showed the highest antifungal activity against all tested fungal strains at all tested lysate concentrations. Furthermore, by increasing the lysate concentration to 3-folds the inhibition increased by about 15 - 20%. About 6 g dry biomass/L of *L. fermentum* were produced using CSL and yeast extract (1% each). The reduction percent in biomass of the tested fungal strains was determined, reduction in biomass against all the tested fungal strains at all tested lysate concentrations (Figure 4a, b and c) with *A. flavus* as the most affected strain (51.6%). Moreover, *L. fermentum* lysate prepared from MRS containing NH_4Cl and CSL resulted in a high and significant inhibition against *A. niger, A. flavus* and *C. albicans* using the disc diffusion method as shown in Figure 5.

An extra experiment was designed to find out the effect of boiled inactive lysate on the growth of test fungal strains. In that case increase in fungal growth indicated that the bacterial lysate consists of peptides with antifungal activity which were probably denaturated by boiling and yielding a solution rich in amino acids and small peptides that stimulate the fungal growth more than control by about 10 - 15% (data not shown).

Fractionation of *L. fermentum* lysate

L. fermentum crude lysate was subjected first to ultra filtration using Vivaspin® through 30 KDa cut off membrane which resulted in two fractions according to their molecular weights. The antifungal activities of the both fractions were tested by disc diffusion assay. As shown in Table 5, the inhibition zones determined for all concentrations of both fractions ranging in their molecular weights from less to more than 30 KDa indicated the poly dispersal nature of the antifungal compounds that ranging in their molecular weights and found on both sides of membrane. A further purification has been done to the crude lysate using DEAE-cellulose column. Results illustrated in Figure 6 for elution profile of the lysate indicated that the lysate contain five peaks (FI to FV). Investigating the yield of the total recovery of about 80% of the applied protein, the first elution fraction (FI was represented in fractions from 3 to 6) revealed the highest fraction in its protein concentration reaching about more than 60% of the original loaded lysate, while from fraction II to V constitute the remaining amount of original protein in crude lysate. A major protein peak was obtained by the first elution which was covered by fractions from 2 to 10. The antifungal activities of the five fractions were examined by disc diffusion assay method. It was observed that the antifungal activity resided mainly in FI against *A. niger, A. flavus* and *C. albicans*. All other fraction peaks

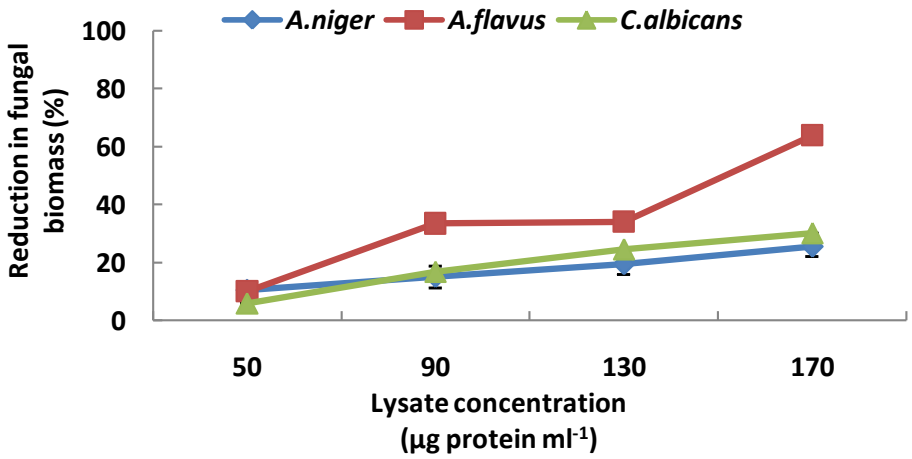

Figure 2. The percentage of reduction in fungal biomass of *A. niger, A. flavus* and *C. albicans,* by *L. fermentum* lysate.

Table 3. Antifungal bioactivity of *L. fermentum* lysate derived by variable carbon sources in MRS medium investigated by measuring the diameter of inhibition zone (disc diffusion assay).

Carbon source*	*L. fermentum* lysate (µg lysate/ml)	Diameter of inhibition zone (mm)		
		A. niger	***A. flavus***	***C. albicans***
Molasses	100	1.1 ± 0.1	1.7 ± 0.2	2.1 ± 0.1
	200	1.5 ± 0.1	1.8 ± 0.2	2.5 ± 0.1
	300	2.1 ± 0.1	2.3 ± 0.2	2.8 ± 0.1
Whey	100	Reduction of the sporulation around the disc	Reduction of the sporulation around the disc	0.0 ± 0.0
	200			
	300			
Wheat bran	100	Reduction of the sporulation around the disc	Reduction of the sporulation around the disc	1.1 ± 0.1
	200			1.4 ± 0.1
	300			1.8 ± 0.1
Lactose	100	1.03 ± 0.1	0.9 ± 0.1	
	200	1.3 ± 0.1	1.1 ± 0.1	0.0 ± 0.0
	300	1.9 ± 0.2	1.5 ± 0.1	

*Also, sucrose and manitol were tested but showed only weak inhibition to growth.

showed low antifungal activity as the reduction of sporulation and growth of the tested fungal strains but no inhibition zone is noticed in the case of *A. niger* and *A. flavus*. On the other hand, fraction no III and IV showed inhibition zone against *C. albicans*. The MIC of FI was determined by disc diffusion assay and it was found to be 25 µg lysate/disc (data not shown).

SDS-PAGE of crude and partially fractified lysate obtained from *L. fermentum*

As shown in Figure 7, the first lane showed the freeze-dried lysate composed mainly of a protein band at an average molecular weight of 20 to 30 KDa. The same was observed with crude lysate as shown in SDS-PAGE graph. The DEAE-cellulose-unbound proteins illustrated in FI lane demonstrated many bands of proteins in the molecular mass range of 20 to 45 KDa. Also, fractions from FII to FV showed mainly 2 protein bands in the range of 20 to 30 KDa, approximately. Peak FI showed the highest activity in relation to the other peaks, due to the high differences in protein concentration basically proteins at 20-30 KDa which where eluted continuously along the 5 fractions.

Haemolytic assay of the *L. fermentum* lysate

Results of toxicity experiments revealed that the total

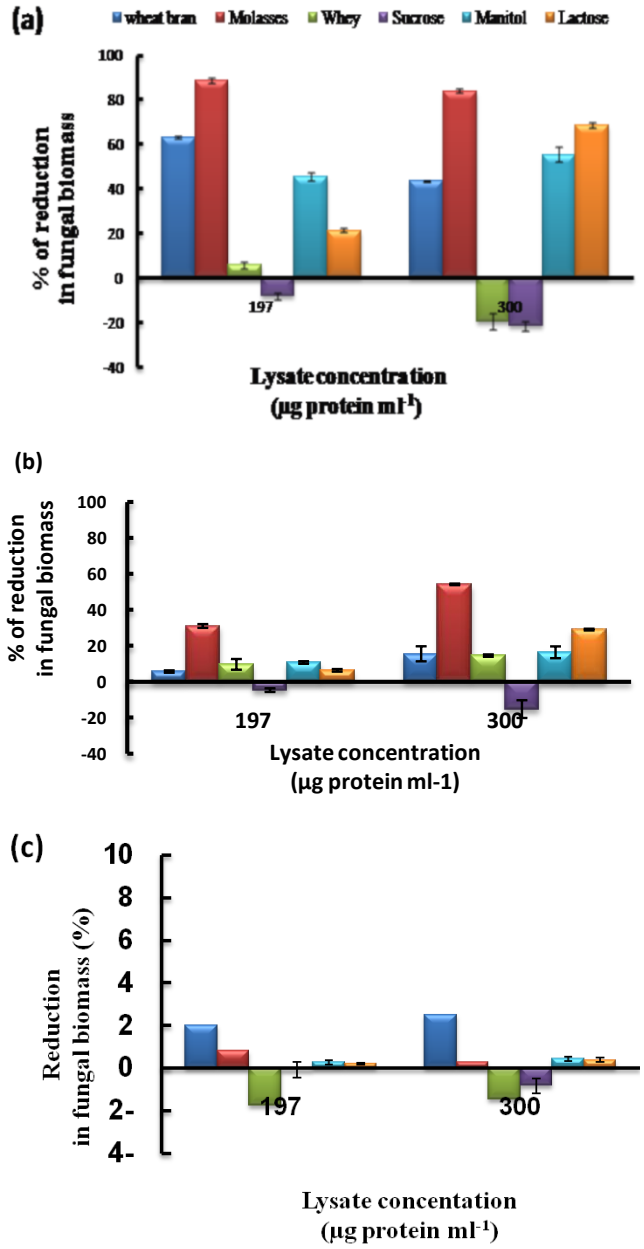

Figure 3. The effect of different carbon sources on the antifungal activity of the resulted bacterial lysate derived from *L. fermentum* against (a) *A. niger*, (b) *A.flavus* and (c) *C. albicans* by measuring the percent of reduction in fungal biomass. ■: Wheat bran; ■: molasses; ■: whey; ■: sucrose; ■: manitol; ■: lactose.

protein lysate of *L. fermentum* investigated in the present study was non toxic to human erythrocytes up to 150 µg lysate/ml. The higher doses exerted insignificant toxicity and only marginal haemolysis (30%) was detected at 300 µg lysate/ml as illustrated in Figure 8. Exoderil, a chemotherapeutic antifungal drug caused lysis of all erythrocytes at a range of 20 to 30 µg exodril/ml. By testing the cytotoxicity of the fractified part that resulted from ultra filtration and fraction FI that was obtained via ion exchange chromatography, it was found that all the concentrations tested showed no significant haemolysis and hence no toxicity to human erythrocytes as compared to

Table 4. Antifungal bioactivity of *L. fermentum* lysate derived by variable nitrogen sources in MRS medium investigated by disc diffusion assay.

Nitrogen source (1%) of each*	*L. fermentum* lysate (µg lysate/ml)	Mean diameter of inhibition zone (mm)		
		A. niger	*A. flavus*	*C. albicans*
NH_4Cl + CSL**	100	1.9 ± 0.1	1.9 ± 0.2	1.4 ± 0.2
	200	2.4 ± 0.2	2.4 ± 0.1	2.1 ± 0.1
	300	2.8 ± 0.2	2.9 ± 0.1	2.5 ± 0.1
Yeast + CSL	100	1.3 ± 0.3	2.1 ± 0.2	2.5 ± 0.1
	200	2.4 ± 0.1	2.7 ± 0.3	2.8 ± 0.2
	300	2.8 ± 0.2	3.3 ± 0.2	3.1 ± 0.1
CSL	100	Reduction of the sporulation around the disc	1.6 ± 0.1	1.5 ± 0.1
	200		1.9 ± 0.2	1.8 ± 0.2
	300		2.0 ± 0.2	1.9 ± 0.1
Yeast	100	Reduction of the sporulation around the disc	1.0 ± 0.1	
	200		1.3 ± 0.2	0.0 ± 0.0
	300		1.4 ± 0.2	

*Also, peptone was tried but showed no result. **Corn steep liquor (CSL).

the chemotherapeutic drug and the lysis buffer.

DISCUSSION

There is presently much active research focusing on the development of target-specific probiotics containing well-characterized bacteria that are selected for their health-enhancing characteristics. In this research, the lysate of *L. fermentum* demonstrated a significant activity against *A. niger, A. flavus* and *C. albicans.* It was found that the MIC of *L. fermentum* lysate was 125 µg/ml against *A. niger* and 62 µg/ml against *A. flavus* and *C. albicans.*

The results also indicated that *A. flavus* and *C. albicans* were more susceptible than *A. niger* to the lethal effect of lysate. While higher concentrations were required to inhibit the growth of *A. niger*. This may be why other investtigators suggested that the surface molecules in different species of pathogens may determine the susceptibility for treatment with bacterial lysate (Yadav et al., 2005b). Similar results were obtained by Magnusson et al. (2003) who screened 1200 isolates of lactic acid bacteria for anti-*Aspergillus* activity and observed a strong inhibitory activity against *A. fumigatus* but several isolates showed reduced antifungal activity after storage and handling.

The results obtained from lysate of *L. fermentum* was similar to that of Yadav et al. (2005a) who indicated that when lysates are prepared from BL21, DH5α, HB101 and XL Blue strains of *Escherichia coli* when examined by using microbroth-dilution assay, these strains exhibited a mild to moderate activity (MICs in µg/ml: DH5α, 62.50; HB101, 125.00; XL Blue, 250.00). They revealed that *E. coli* reported a very important medicinal value which was also supported by Matricardi et al. (2003). The supernatant of *Streptomyces thermonitrificans* showed better anti-*Aspergillus* activity (MIC, 62.5 µg/ml) than lysate (MIC, 125 µg/ml) Yadav et al. (2005a, 2007). *C. albicans* remains the most susceptible of the yeasts studied for fluconazole and itraconazole when compared with non *C. albicans* (Panizo et al., 2009).

No studies have been carried out on the optimization of lysate production from bacteria, however, there was a research by Leal et al. (2002) showing that bacteriocins production changes dramatically upon altering of environmental conditions and optimum production may require a specific combination of environmental parameters. The best result of biomass yield was obtained in MRS medium supplemented with 2% wheat bran (10 g/l) of *L. fermentum*. Wheat barn is a good source of proteins, starch and some vitamins, which confers excellent substrate for growth and reduce the possibility of biosynthesis of secondary metabolites in the relatively short period of incubation. Also, the best nitrogen source that resulted in a large biomass yield (5.8 g/l) of *L. fermentum* was obtained from MRS medium supplemented with 1% yeast extract and 1% CSL furthermore. The significance of this funding lies in the low cost of molasses, yeast extract and CSL. As compared to glucose, peptone and beef extract, the aim was achieved in this research. CSL is of great importance in the production of antibiotics where it is considered as a source of free amino acids, many vitamins and minerals that are incorporated in protein synthesized ribosomally and non ribosomally (Zabriskie et al., 1982).

The fractifaction of *L. fermentum* lysate yielded 5 fractions of protein peaks. The protein profile of the crude lysate gave large number of bands when run on SDS–PAGE and the fractified peak with the highest antifungal activity showed many protein bands in the molecular

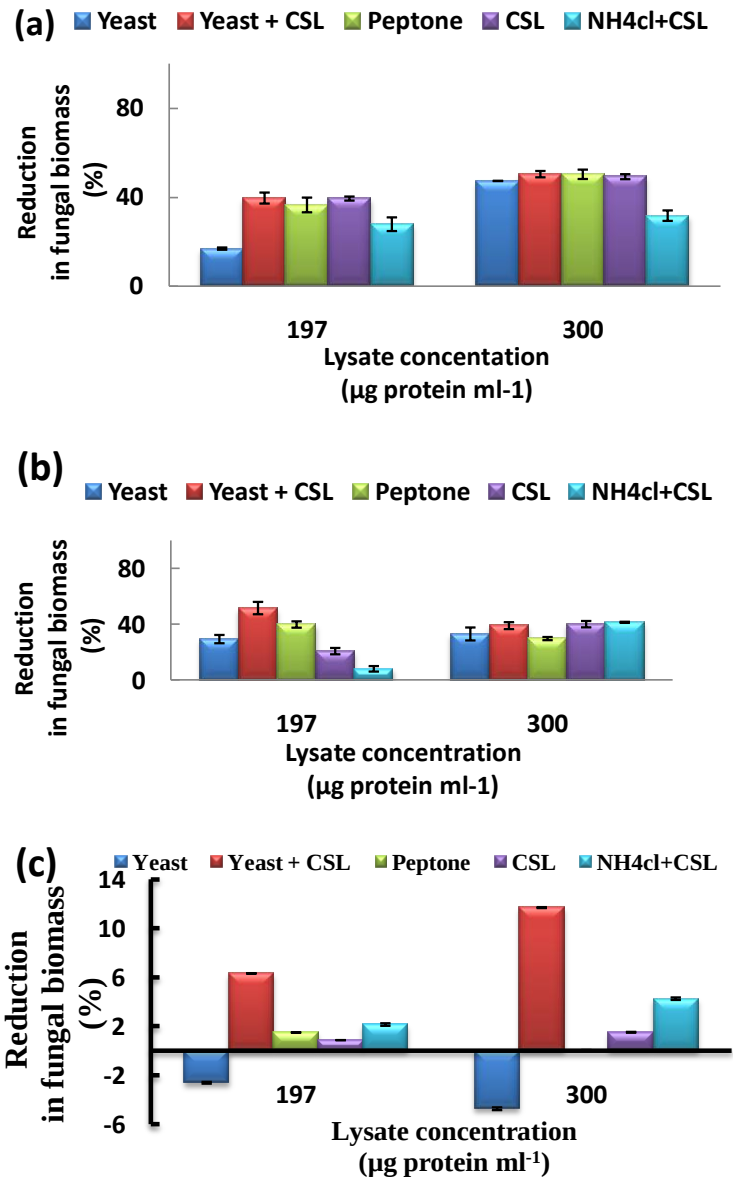

Figure 4. Antifungal activity of the lysate derived from *Lact. fermentum* grown on different nitrogen sources tested against (a) *A. niger,* (b) *A. flavus,* (c) *C. albicans* by measuring the percent of reduction in fungal biomass.

mass range of 20 – 45 KDa. This result was in accordance with that observed by Yadav et al. (2005a and 2007) showing that the fractifaction of *E. coli* BL21 lysate showed 5 fractions of protein. The protein profile of crude *E. coli* BL21 lysate and the active fraction with antifungal activity showed that crude lysate gave various bands when run on SDS-PAGE, while the active fraction with antifungal activity demonstrate two major bands in the molecular mass range of 29 to 43 KDa. The MIC of the active fraction was found to be 3.90 µg/ml against *A. fumigatus* by microbroth dilution assay while by disc diffusion method its MIC was 1.25 µg/disc.

In the present study, crude lysate was non toxic up to a test concentration of 200 µg/ml on the other hand, the fractified fraction with antifungal activity FI showed no toxicity with all tested concentrations up to 2000 µg/ml against erythrocytes, as compared to this results with that obtained by Yadav et al. (2005a and 2007) who showed that *E. coli* BL21 total lysate and completely purified fraction with antifungal activity PPEBL21 were non toxic

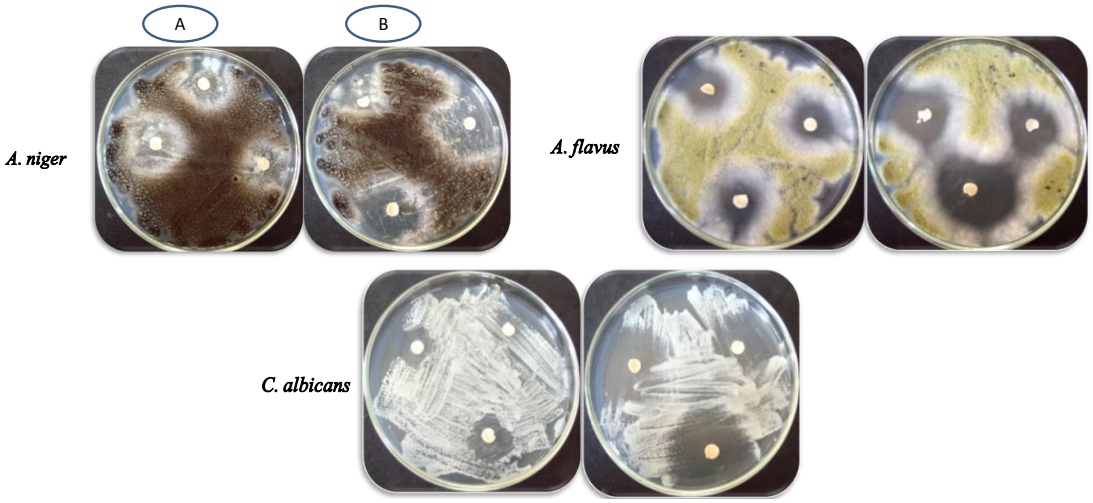

Figure 5. Inhibition of fungal growth by disc diffusion assay method using 100, 200 and 300 µg ml of *L. fermentum* lysate prepared from A) MRS-NH_4Cl with CSL, B) MRS-yeast extract with CSL.

Table 5. Antifungal bioactivity of the fractified part of lysate against *A. niger, A. flavus* and *C. albicans* studied by measuring the diameter of inhibition zone (disc diffusion assay).

***L. fermentum* lysate (µg disc^{-1})**		**Mean diameter of inhibition zone (mm)***		
		A. niger	***A. flavus***	***C. albicans***
M.wt. over 30 KDa	62.25	1.8 ± 0.6	4.5 ± 0.7	3.3 ± 0.3
	125.00	1.9 ± 0.2	4.1 ± 0.1	3.5 ± 0.6
	250.00	1.9 ± 0.2	4.3 ± 0.4	3.8 ± 0.0
	350.00	2.0 ± 0.1	4.7 ± 0.2	5.0 ± 0.0
M.wt. lower than 30 KDa	62.25	1.3 ± 0.2	2.8 ± 0.4	2.3 ± 0.3
	125.00	1.5 ± 0.3	3.7 ± 0.3	3.0 ± 0.2
	250.00	1.8 ± 0.3	4.1 ± 0.1	3.8 ± 0.3
	350.00	1.9 ± 0.1	4.3 ± 0.1	4.2 ± 0.3

*Mean diameter of inhibition zone using fraction of bacterial lysate obtained by ultra filtration using Vivaspin® 30 KDa cut off membrane.

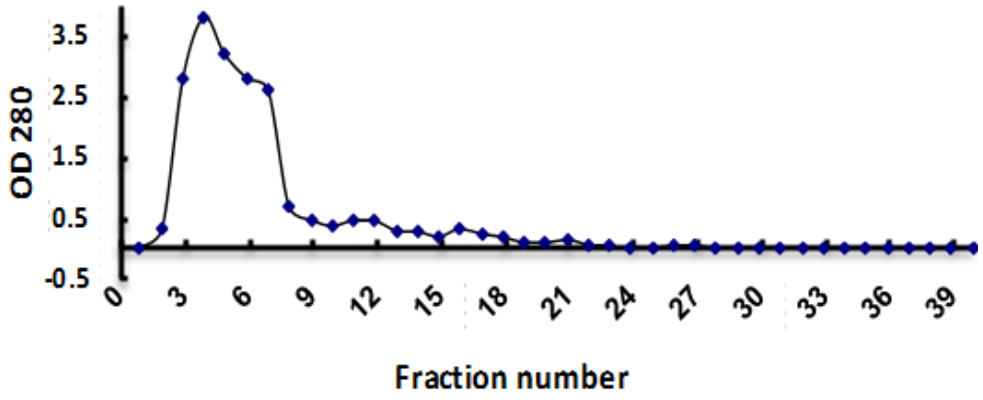

Figure 6. Elution profile of *L. fermentum* lysate on DEAE-cellulose represented five peaks from FI to FV: FI (3-5), FII (6-8), FIII (9-12), FIV (15-17), FV (20-22). OD, optical density.

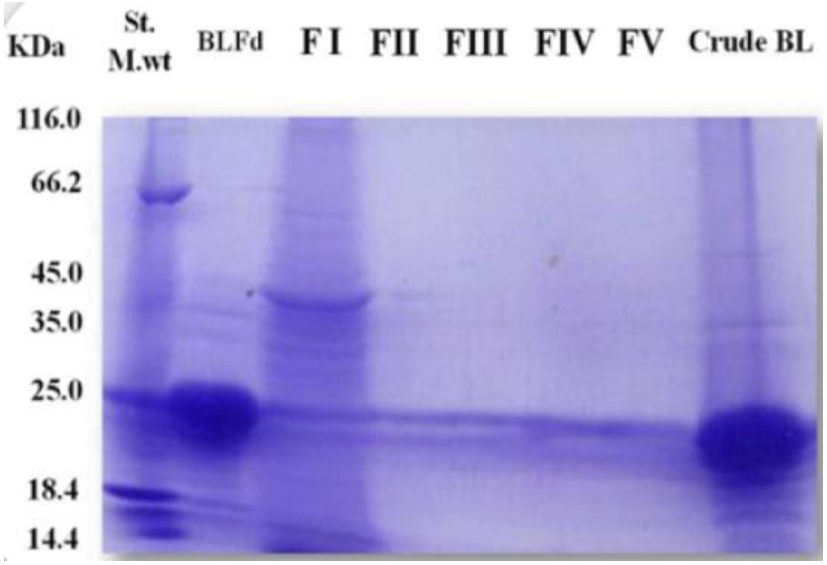

Figure 7. SDS-PAGE showing the protein profile of a purified (FI, FII, FIII, FIV, FV), freeze dried bacterial lysate (BLFd) and crude (BL) lysate obtained from *L. fermentum* previously cultivated under optimum conditions for lysate production.

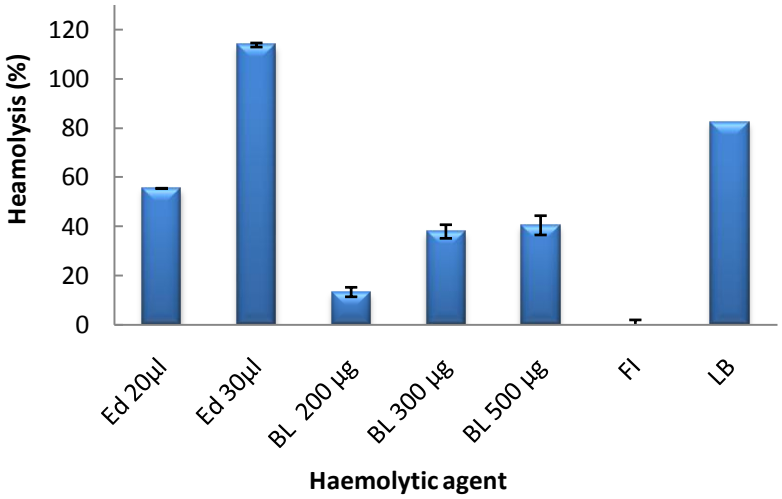

Figure 8. Cytotoxicity of lysate obtained from *L. fermentum* previously cultivated under optimum conditions investigated by lysis of healthy human erythrocytes using the haemolytic assay. Ed, Exoderil drug; BL, total bacterial lysate; FI; peak 1, LB, lysis buffer.

up to a test concentration of 1250 µg/ml to human erythrocytes. These results gave evidence that the partially purified *L. fermentum* lysate is totally safe for producing potent antifungal compound.

Conclusion

There are few studies in the emerging field of bacterial lysates which provides evidence that the lysate of the *L. fermentum* which was naturally occurring probiotic strains in human dairy food had a potential and promising antifungal activity. As compared to chemotherapeutic antifungal drugs, it had extremely very low toxicity to human cells, therefore, it could be an important source of biologically active and less toxic compounds useful for developing new better antifungal preparations.

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Alteration of BHK-21 cells proteome after foot-and-mouth disease virus infection

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Foot-and-mouth disease is a highly contagious viral illness of wild and domestic cloven-hoofed animals. The complex relationship of FMDV and with the host cells leads to its replication and spread. BHK-21 cell line is an *in vitro* model for FMDV infection and is commonly used for viral seed preparation. In order to better understand the molecular basis of this relationship, a proteomics study on baby hamster kidney cells infected with FMDV was performed. The differential proteomes of BHK-21 cells, with and without BHK-21 infection, were analyzed with two-dimensional gel electrophoresis (2-DE) followed by MALDI-TOF/TOF identification. Mass spectrometry identified 30 altered protein spots (19 up-regulated, 9 down-regulated and 2 viral protein spots), which included metabolic processes proteins, cytoskeletal proteins, microfilament-associated proteins, stress response proteins and FMD viral proteins. Western blot analysis further confirmed the differential expression of protein NME-2 in the proteomic profiles. Subcellular location demonstrated NME2 protein was distributed in BHK-21 cell cytoplasm and nucleolus. Thus, this work provides useful proteinrelated informations to further understand the underlying pathogenesis of FMDV infection.

Key words: Foot-and-mouth disease virus, BHK-21 cells, comparative proteomics, 2-DE, NME2.

INTRODUCTION

Foot and mouth disease virus (FMDV) causes a highly infectious disease of cloven-hoofed animals that has significant global socioeconomic impact (Schley et al., 2012). Although FMD does not result to high mortality in adult animals, the disease has negative effects, including decrease in milk production, weight loss and loss of draught power, resulting in a loss in productivity for a considerable time (Wang et al., 2012). However, mortality can be high in young cloven-hoofed animals, where the virus can affect the heart. In countries where FMD is endemic the disease results in enormous losses and it is ranked in the top ten livestock diseases for cattle and pigs in terms of impact on the poor globally. FMDV belongs to the *Picornaviridae* family and has single-stranded, positive-sense RNA, with seven serotypes and its genome has one large open reading frame (ORF), which encodes a precursor protein (Bachrach, 1968; Leforban, 1999; Martinez-Salas et al., 2008). After processing by proteases, the precursor protein is split into single proteins, including four structural proteins, that is, VP1, VP2,VP3 and VP4, and eight nonstructural proteins, that is, L^{pro}, 2A, 2B, 2C, 3A, 3B, $3C^{pro}$ and $3D^{pol}$ (Rueckert, 1996). The BHK-21 cell line provides ideal cells for researching the infectious and pathogenic mechanism of

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Abbreviations: CAN, acetonitrile; **BHK,** baby hamster kidney; **CPE,** cytopathic effect; **ER,** endoplasmic reticulum; **FMDV,** foot and mouth disease virus; hpi, hours pos-tinfection; **MS,** mass spectrometry; **TFA,** trifluoroacetic acid.

FMDV (Huang et al., 2011; Mitev and Tekerlekov, 1973; Ubertini et al., 1967).

Proteomic technology that couple two-dimensional electrophoresis (2-DE) and mass spectrometry (MS) are widely used (Aebersold and Mann, 2003; Blackstock and Weir, 1999), it is a powerful tool for providing insights into pathogenesis, diseases biomarkers, and the prevention of disease (Hanash, 2003; Misek et al., 2011; Wright Jr and Semmes, 2003). Proteomic alteration in infected host cells have been studied in major pathogenic animal disease viruses, including classical swine fever virus (PK-15 cells were used) (Sun et al., 2008), African swine fever virus (Vero cells were used) (Alfonso et al., 2004), porcine reproductive and respiratory syndrome virus (pulmonary alveolar macrophage were used) (Zhang et al., 2009), infectious bursal disease virus (chicken embryo fibroblasts were used) (Zheng et al., 2008) and rabies virus (BHK-21 cells were used) (Zandi et al., 2009).

In the present study, a baby hamster kidney (BHK) cell line (BHK-21) was infected with FMDV serotype Asia1 and the proteome pattern of the cell was investigated 12 h post infection. This simple mammalian cell infection model was selected to analyze the direct effect of FMDV on cell protein machinery free from influences of external stimuli. A total of 30 differentially expressed protein spots were identified. We found that viral proteins, host cell cytoskeletal proteins, microfilament-associated proteins, stress response proteins were the main proteins with significant altered expression profile. Further analysis of these data provides clues to understanding the replication and pathogenesis of FMDV and the virus-host interactions.

MATERIALS AND METHODS

Cell culture, virus inoculation

FMDV serotype Asia1 was provided by the National Foot-and-Mouth Disease Reference Laboratory in Lanzhou, China. BHK-21 cells were cultured in modified Eagle's medium (MEM, HyClone) supplemented with 10% fetal bovine serum, 100 U/mL penicillin G and 100 mg/mL Na streptomycin sulfate at 37°C in 5% CO_2 until they formed a monolayer. After washing three times with PBS (pH 7.4), the cells were inoculated with FMDV serotype Asia1. Uninfected cells were incubated in MEM as a mock-infected control. Fluorescence quantitative RT-PCR was used to valuate FMDV reproduction interval two hours.

Protein extraction, 2-DE gel staining and image analysis

The infected and uninfected cells were mechanically scraped and collected into centrifuge tubes 12 hpi. After three cycles of washing with ice-cold PBS (pH 7.4) and centrifugation (8000 x*g* for 5 min), harvested cells were lysed with lysis buffer containing 7 M urea, 2 M thiourea 4% CHAPS, 20 mM Tris, 50 mM DTT, 0.5% IPG buffer and 1 mM FMSF at a volume ratio of 1:20. After 2 h on ice, DNase and RNase were added to the mixture at final concentrations of 20 U/mL and 0.25 mg/mL, respectively, and nucleic acids were degraded on ice for 1 h. After centrifugation at 16000 x*g* for 20 min at 4°C, the supernatants were collected and the protein concentration was determined using a Quant kit (Bio-Rad).

2-DE using 7 cm IPG strips at nonlinear pH 3-10 (Bio-Rad) in the first dimension isoelectric focusing (IEF) were performed. The IPG strips were rehydrated with 150 µL of rehydration buffer (8 M urea, 4% CHAPS, 50 mM DTT, 0.2% IPG buffer pH 3~10 NL, and trace amount of bromophenol blue) containing 100 µg of protein samples, before staining with Coomassie brilliant blue. Active rehydration was achieved by applying 50 V for 12 h. IEF was carried out at 18°C in a Protean IEF cell (Bio-Rad), where the current was limited to 50 mA/strip with the following voltage program: 500 V linear for 30 min, 1000 V rapid for 30 min, 4000 V linear for 3 h, then 4000 V constant for a total of 20 000 Vh. After IEF, the IPG strips were equilibrated by soaking for 15 min in 50 mM Tris-HCl, pH 8.8, 6 M urea, 2% SDS, 30% glycerol, 2% (w/v) DTT, and a trace of bromophenol blue. This was followed by another 15 min in the same solution, which contained 2.5% (w/v) iodoacetamide instead of DTT. The second-dimensional separation was performed using 12% homogeneous SDS polyacrylamide gel. Electrophoresis was carried out at a constant current of 80 V/gel for 5 min, followed by 200 V/gel, until the dye reached the bottom of the gel.

The gels were stained with Coomassie brilliant blue and scanned at a resolution of 500 dots/inch using a scanner (GS-800 Calibrated Densitometer). Spot detection, matching and quantitative intensity analysis were performed using the PDQuest 2-D analysis program (Bio-Rad). A relative comparison of intensity abundance was performed between FMDV-infected and mock-infected groups (three replicate samples for each group) using the Student's *t*-test. Infected /uninfected expression intensity ratios higher than 2.0 ($p \leq 0.05$) or less than 0.5 ($p \leq 0.05$) were set used as threshold values to detect significant differences.

Enzymatic digestion, MALDI-TOF/TOF MS and database search

Significant differential protein spots were excised manually from Coomassie-stained gels and washed with 100 µL 50% v/v acetonitrile (ACN) in 25 mM ammonium bicarbonate for 1 h. After dehydration with 100% v/v ACN for 20 min, the gel pieces were dried thoroughly with a speedVac concentrator (Thermo Savant, U.S.A) for 30 min. The dried gel particles were rehydrated for 45 min at 4°C with 2 µL trypsin (Promega, Madison, WI) in 25 mM ammonium bicarbonate and then incubated for 12 h at 37°C. The resulting peptides were extracted three times using 8 µL aliquots of 5% trifluoroacetic acid (TFA) in 50% ACN for 1 h at 37°C and dried by vacuum centrifugation.

The peptide mixtures were redissolved in 0.8 uL of matrix solution (α-cyano-4-hydroxycinnamic acid (Sigma) in 0.1% TFA and 50% ACN) and then spotted onto a MALDI plate. Samples were allowed to air-dry and analyzed using a 4700 MALDI-TOF/TOF Proteomics Analyzer (Applied Biosystems, Foster City, CA). Trypsin-digested peptides of myoglobin were added to the six calibration spots on the MALDI plate to calibrate the mass instrument in the internal calibration mode. The UV laser was operated at a 200-Hz repetition rate with a wavelength of 355 nm. The accelerated voltage was operated at 20 kV. All acquired spectra of samples were processed using the 4700 ExploreTM program (Applied Biosystems) in the default mode. Parent mass peaks with a mass range of 700-3200 Da and a minimum signal to noise ratio of 20 were selected for tandem TOF/TOF analysis.

Combined MS and MS/MS spectra were submitted to MASCOT (Version 2.1, Matrix Science, London, UK) using the GPS explorer program (Version 3.6, Applied Biosystems) and searched using the following parameters in the National Center for Biotechnology Information non-redundant (NCBInr) database (release date, March 18, 2006): taxonomy of bony vertebrates or viruses, trypsin digest with one missing cleavage, no fixed modifications, MS tolerance of 0.2 Da, MS/MS tolerance of 0.6 Da, and possible oxidation of methionine. Known contaminant ions (human keratin and tryptic autodigest peptides) were excluded. A total of 4,736,044 sequences

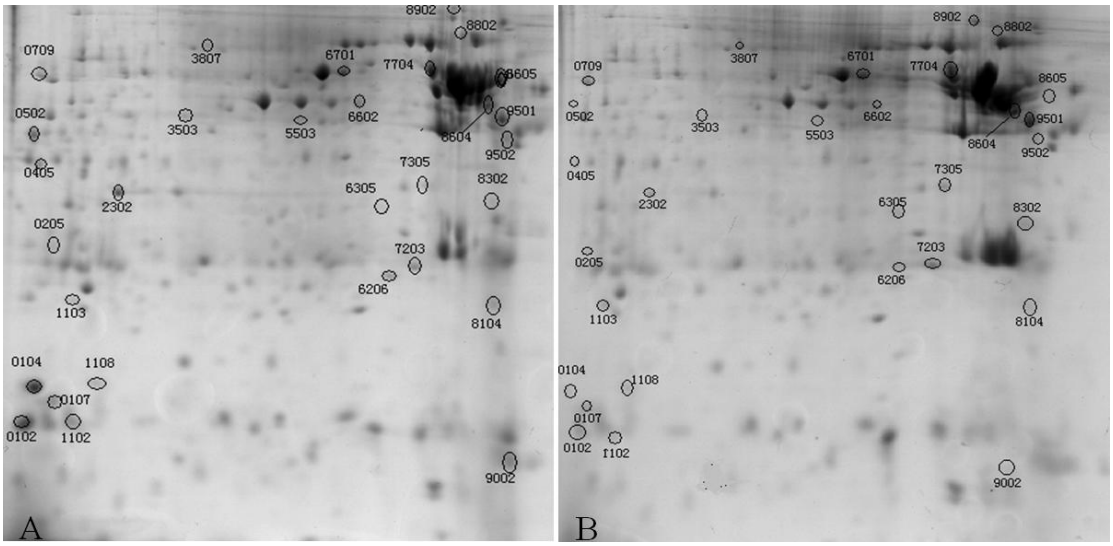

Figure 1. 2-DE analysis of FMDV-infected BHK-21 cells at 12 hpi. Circles show the protein spots from which the proteins were isolated. (A) FMDV infected cells; (B) non-infected cells

and 1,634,373,987 residues were actually searched in the database. MASCOT protein scores (based on combined MS and MS/MS spectra) > 72 were considered statistically significant ($p \leq 0.05$). We accepted individual MS/MS spectra with a statistically significant (confidence interval ≥ 95%) ion score (based on MS/MS spectra). To eliminate the redundancy of proteins that appeared in the database with different names and accession numbers, we singled out a single protein member belonging to the species *Gallus* or that with the highest protein score (top rank) from a multiprotein family.

Data analysis

Protein classification was conducted using Gene Ontology Annotation (GOA; http://www.ebi.ac.uk/goa/), according to molecular functions and biological processes. The subcellular location of different proteins was predicated with PSORT (http://psort.hgc.jp/).

Western blot analysis

A total of 50 mg proteins from FMDV-infected and mock-infected BHK-21 cells at 12 h post infection (hpi) were mixed with an equal volume of SDS-PAGE loading buffer and boiling for 5 min. After separation using 12% sodium dodecyl sulfate polyacrylamide gels, proteins were electro-transformed onto PVDF membranes and blocked nonspecifically using 1% BSA in 0.01 mol/L PBS, pH 7.4, for 2 h at room temperature. Membranes were then incubated successively for 2 h at ambient temperature with mouse monoclonal antibodies to NME2 (Abcam, Cambridge, U.K.). After three 15 min washes with PBST, the membranes were further incubated with rabbit anti-mouse IgG conjugated with horseradish peroxidase (Sigma, St. Louis, MO) (1:5000 dilution in 1% BSA in 0.01 mol/L PBS, pH 7.4) at room temperature for 2 h. Reactive protein stripes were visualized using Super Signal West Pico Chemiluminescence Substrate (Pierce Biotechnology, Inc., Rockford, IL) after three times washes with PBST. Equal protein loading was confirmed by exposure of the membranes to anti β-actin antibody.

Subcellular localization of NME2 in BHK-21 cells

Primers for the NME2 gene were designed based on reference sequences published in GenBank and then synthesized (Takara Co. Ltd). The forward primer sequence was 5′-CCCAAGCTTATGGCCAACCTCGAGCGTACCTT-3′ and the reverse primer sequence was 5′-CGGGATCCCTCATACACCCAGTCATGGGCA-3′. Total RNA from BHK-21 cells was extracted using an RNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The NME2 gene was amplified by reverse transcription polymerase chain reaction (RT-PCR) and cloned into the pEGFP-N1 vector (Invitrogen, USA) directionally with the restriction endonucleases *Bam*HI and *Hind*III. The recombinant plasmid was identified by PCR, restriction enzyme digestion analysis, and sequencing.

Empty pEGFP-N1 and recombinant plasmid pEGFP-NME2 were transfected into BHK-21 separately using LipofectamineTM 2000, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). After transfection for 12 h, cells were washed twice with PBS and fixed with cold acetone/methanol (1:1) for 20 min at -20°C. Cell nuclear staining was performed with 4′,6-diamidino-2-phenylindole (Sigma) for 5 min and washed three time with PBS. Subcellular location of NME2 was observed using a Zeiss LSM510 laser confocal microscope.

RESULTS

All gels provided high resolution separation of proteins, with each detecting over 800 protein spots (Figure 1). On the basis of the average intensity ratios of protein spots, a total of 30 protein spots were identified, including 19 significantly upregulated protein spots (ratio infection/control ≥2, $p \leq 0.05$), 9 significantly downregulated protein spots (ratio infection/control ≤ 0.5, $p \leq 0.05$), and 2 FMDV proteins.

The combined MS and MS/MS analysis with MASCOT database searching successfully identified 30 proteins, including 19 upregulated and 9 downregulated intracellular proteins, and 2 FMDV proteins (Table 1). The cellular proteins identified were involved mainly in five groups, which included morphogenesis, protein synthesis, meta-

Table 1. List of the differentially expressed protein spots in FMDV-infected BHK-21 cells identified by MALDI-TOF or MALDI-TOF/TOF.

Spot no.[a]	Protein name	Abbr.	Accession no.[b]	MW (Da)	pI	Protein score[c]	Sequence coverage[d]
Significantly up-regulated proteins							
5503	beta-actin (aa 27-375)	ACTB	gi|49868	39446	5.78	190	25
3503	gamma-actin	ACTC	gi|809561	42053	5.23	191	28
8605	vimentin	VIM	gi|2078001	51590	4.96	587	56
9502	vimentin	VIM	gi|2078001	51590	4.96	305	48
8802	glucose-regulated protein precursor	GRP78	gi|254540166	68569	5.15	210	29
0205	enoyl-CoA hydratase, mitochondrial precursor	ECHMP	gi|29789289	31853	8.76	118	23
8902	unnamed protein	NP	gi|74228123	97243	5.39	65	12
0502	fructose-bisphosphate aldolase A isoform 2	FBAA2	gi|6671539	39787	8.31	206	34
0405	mitochondrial malate dehydrogenase 2	NAD2	gi|89574115	32113	7.70	81	19
6602	hypothetical protein LOC433182	LOC	gi|70794816	47931	6.63	99	17
0709	ATP synthase	ATPase	gi|148677501	54675	8.24	348	40
0107	Non-metastatic protein2	NME2	gi|154550673	16460	7.77	187	25
0102	Peptidyl isomerase A	PA	gi|6679437	18131	7.74	174	42
1102	Peptidyl isomerase A	PA	gi|6679439	18131	7.74	233	36
0104	cofilin-1	C1	gi|6680924	18776	8.22	170	54
1108	cofilin CRA-b2	CCB2	gi|148704795	19300	7.59	183	40
9002	mCG140959, isoform	mCG1	gi|148692630	17959	4.52	228	21
8302	mCG22236	mCG2	gi|148683218	28993	4.71	88	27
8104	mCG10592, CRA-c	mCG1C	gi|148703873	16581	5.23	108	19
Significantly down-regulated proteins							
1103	peroxiredoxin 1	PXD1	gi|123230137	19669	6.28	141	32
7203	gamma actin	ACTC	gi|123298587	32941	5.15	253	25
7305	Pyruvate dehydrogenase	PDE1	gi|18152793	4387	6.61	117	30
6305	CP isoform 1	CPI1	gi|4826695	29463	6.14	103	46
3807	isoform C	IC	gi|161760667	65508	6.25	101	21
6701	disulfide-isomerase A3	DIA3	gi|112293264	57103	5.78	183	26
9501	vimentin	VIM	gi|2078001	51590	4.96	443	47
8604	vimentin	VIM	gi|2078001	51590	4.96	381	60
7704	Hspd1 protein	Hsp	gi|76779273	59559	8.09	474	39
Viral proteins of FMDV							
2302	nonstructural protein 2C	2C	gi|16902989	36281	7.74	186	21
6206	precursor protein P1	P1	gi|15419535	80620	6.44	202	12

[a]Spot no. is the unique sample spot protein number that refers to the labels in Figure 1. [b]Accession no. is the MASCOT result of MALDI-TOF/TOF searched from the NCBInr database. [c]Protein score (based on combined MS and MS/MS spectra) were from MALDI-TOF/TOF. [d]Sequence coverage (%) is the number of amino acids spanned by the assigned peptides divided by the sequence length.

metabolism and stress response. The proteins were classified according to their biological function (Figure 2A) and subcellular location (Figure 2B). To confirm the dynamic changes in proteins during FMDV infection, we performed a western blot analysis of the NME2, where β-actin was used as an internal control. NME-2 spot number was labelled 0107 in Table 1 and Figure 1. NME2 expression was significantly upregulated at 12 hpi when compared with uninfected cells (Figure 3). This result was consistent with the 2-D PAGE analysis. Subcellular distribution of NME2 indicated that the NME2 protein was distributed in the cytoplasm and nucleolus (Figure 4).

DISCUSSION

Increasing evidence emphasizes comparative proteomics to screen the differentially expressed proteins associated with host cellular pathophysiological processes of virus infection (Maxwell and Frappier, 2007). BHK-21 cells was

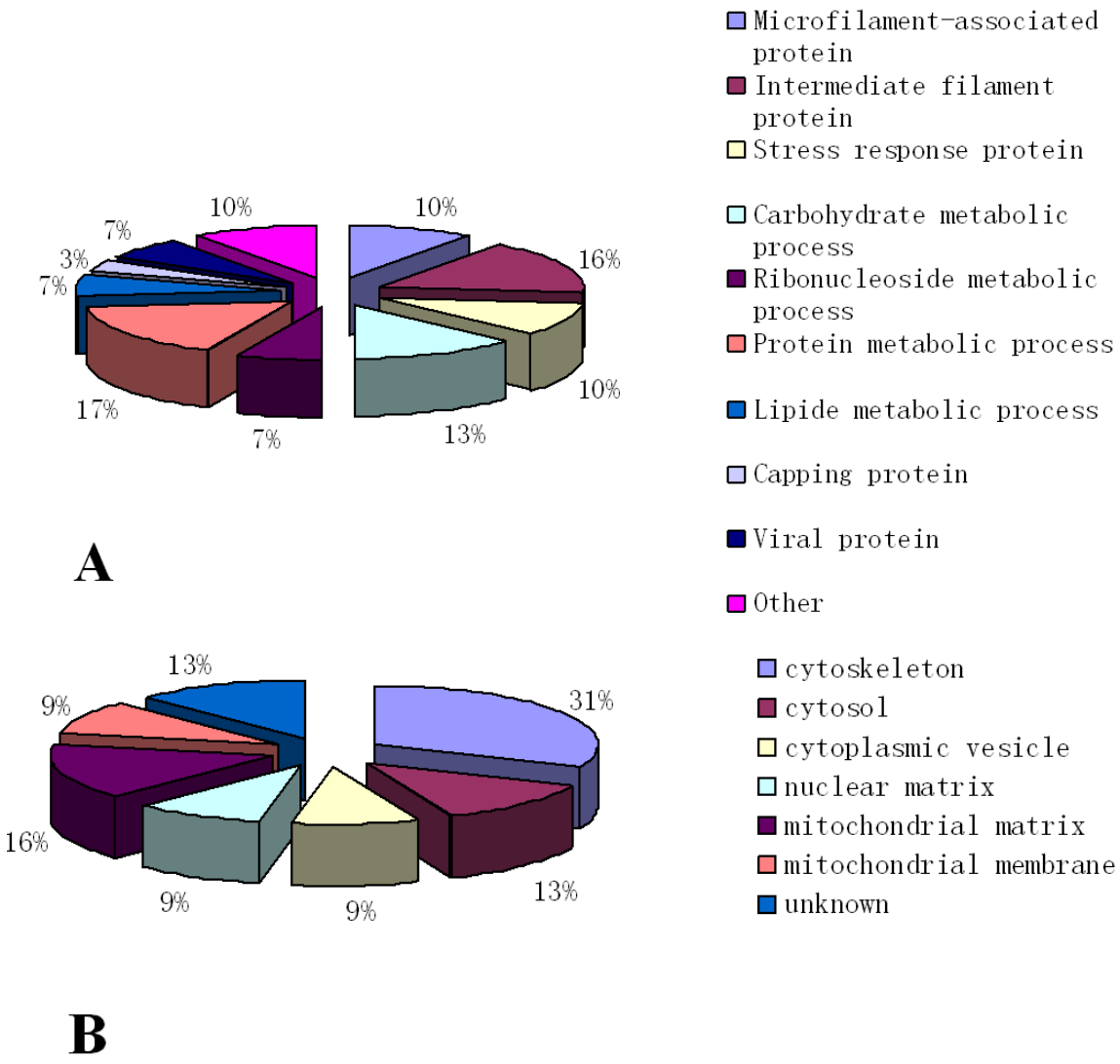

Figure 2. Classification of differentially expressed proteins in FMDV-infected BHK-21 cells, according to the function and subcellular locations of the altered proteins.(A) Functional classification of the affected protein spots. (B) Subcellular location of altered protein spots.

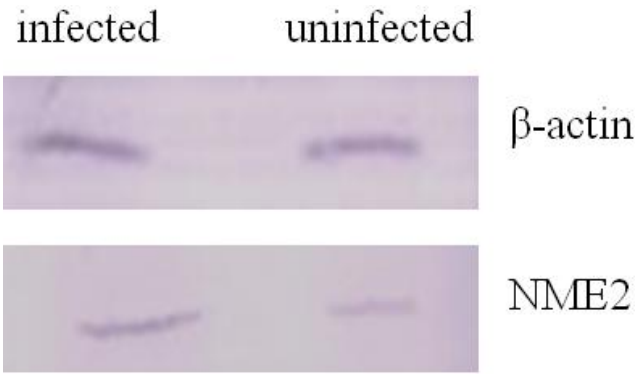

Figure 3. Confirmation of a differentially expressed protein (NME2) in FMDV-infected BHK-21 cells by western blot analysis. β-Actin was used as an internal control to normalize the quantitative data.

often chosen as a simple *in vitro* model for analysis of the direct viral influences on host cell protein machinery, and routinely used for FMDV pathogenic research (Anil et al., 2012; Chen et al., 2004; Huang et al., 2011). Reproduction of FMDV reached plateau period and CPE is the most obvious during 12 h infection. So, the time at 12 h post infection was selected. From the literature, it appears that very few studies have been performed to analyze the interplay between FMDV and host cells using proteomics analysis. In our study, we obtained an overview of the altered protein expression of host cells responding to FMDV infection (Figure 1). The identified cellular proteins function in cytoskeleton organization, metabolic processes proteins, microfilament-associated proteins and stress response proteins (Table 1).

According to the predicted function classification, the

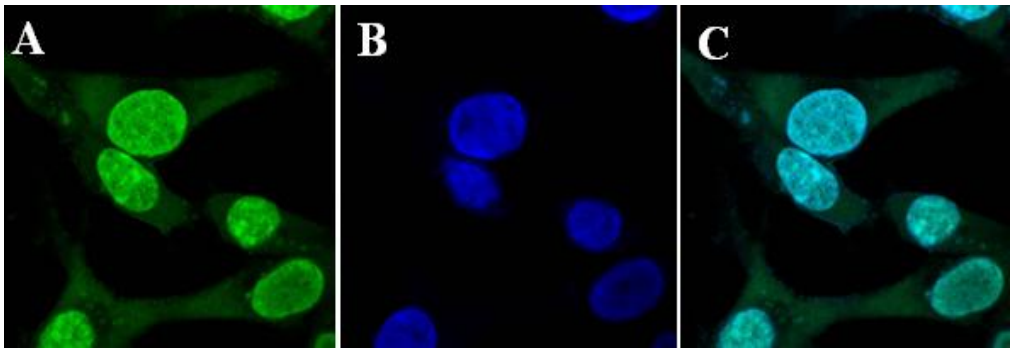

Figure 4. Subcellular distribution of NME2 proteins in BHK-21 cells. (A) Expression of GFP- NME2 fusion protein in the cytoplasm and nucleolus of BHK-21cells. (B) DAPI staining of the nucleolus. (C) A integrated with B.

following proteins accounted for the total differentially expressed proteins: microfilament-associated proteins (10%), intermediate filament protein (16%), stress response proteins (10%), process proteins (44%), capping proteins (3%), FMDV proteins (7%), and other proteins (10%) (Figure 2A). The subcellular localizations of the identified proteins were as follows: cytoskeleton (31%), cytosol (13%), cytoplasmic vesicle (9%), nuclear matrix (9%), mitochondrial matrix (16%), mitochondrial membrane (9%), unknown distribution (13%) (Figure 2B).

The cytoskeletal proteins were the most abundant ones among the significantly altered proteins. These cytoskeletal proteins included capping protein (CP) of actin filament and vimentin. Vimentin is a major component of type III intermediate filaments, which involve cell integrity maintenance, cell movement, cell division process and scaffold structure (Chou et al., 2003). Actin is the major component of microfilaments and essential for a large range of cell functions, including cell division, migration, junction formation, chromatin remodeling, transcriptional regulation, vesicle trafficking and cell shape regulation (Perrin and Ervasti, 2010). We identified two vimentin-related and three actin-related proteins which appeared differentially expressed after infection (Figure 1). These fragments could be products of vimentin and actin cleavage, considering the observed MW and pI (Table 1). Therefore, precise function of vimentin and actin cleavage and specific rearrangement of cell architecture during FMDV infection could be important for better understanding of the FMDV replication process.

Glucose-regulated protein 78 (GRP78) is a stress response protein and a major endoplasmic reticulum (ER) chaperone protein, which is essential for protein quality control in the ER and a central regulator of the unfolded protein response (UPR). The induction of GRP78 is well established as a marker of ER stress (Chen and Lee, 2011). Previous studies have shown that GRP78 is an intracellular antiviral factor against hepatitis B virus (Ma et al., 2009), while GRP78 is also necessary for DENV antigen production and/or accumulation as a chaperone in viral antigen production (Wati et al., 2009). GRP78 expression was upregulated in this study, so we may infer that GRP78 can protect BHK-21 cells from FMDV infection.

Apoptosis is an active process that involves gene activation, expression and regulation (Elmore, 2007; Rasheva and Domingos, 2009). Cellular apoptosis is protective response because it eliminates infected cells (Brereton and Blander, 2010). BHK-21 cells infected with FMDV cause apoptosis, which was confirmed by Bin (2007). In this study, two cell apoptosis-related proteins were identified including PXD1 and NME2. PXD1 is an antioxidant and molecular chaperone that can be secreted by tumor cells (Riddell et al., 2010) and it can catalyze peroxidase reduction of H2O2, organic hydroperoxides and peroxynitrite (Rhee, 2006; Rhee et al., 2005). PXD1 has a role against apoptosis and it is increased and pro- vided enhanced protection against the apoptosis (Berggren et al., 2001). It may have a negative role in ASK1-induced apoptosis (Kim et al., 2008). NME2 is also known as nucleoside diphosphatekinases (NDPK) that are implicated in tumorigenesis as suppressors of tumor metastasis (Leone et al., 1991), it is an isoform of multifunctional proteins involved in a variety of cellular activities including proliferation, development, adhesion and differentiation (Lombardi and Mileo, 2003). As a specific binding protein of Diva and Bcl2L10 and identified as a new biological function, NME2 overexpression induced apoptosis while the depletion led to an increase in Diva's apoptotic activity (Kang et al., 2007). High levels of NME2 could enhance apoptosis in synergy with other metastasis suppressors such as TIP30 (Xiao et al., 2000). Apoptosis is the result of interactions between FMDV and BHK-21 cells. NME2 can promote apoptosis, while PXD1 inhibit apoptosis. In our study, it can be found that NME2 differentially upregulated while PXD1 remarkably down-regulated (Figure 1). It can be concluded that FMDV provide favorable environment for its own replication by regulating NME2 and PXD1 protein expression in BHK-21 cells.

Unexpectedly, we could not detect and identify the other FMDV proteins in our experiment except 2C and P1, the well known cellular substrates of the viral proteinases (e.g., eIF4G) which were not modified in infected cells,

this could be due to the sample preparation method we used and also to the limited resolving power of 2-DE.

This study adopted a gel-based proteomics approach to probe the changed proteins in FMDV infected BHK-21 cells. It is noteworthy that the comparative proteomics approach allowed for the initial identification of 30 altered cellular proteins during FMDV infection and showed that most of the altered cellular proteins appear to have roles in revealing the viral pathogenesis. Clearly, further large scale studies are necessary to understand the roles of the differentially expressed cellular proteins in FMDV infection.

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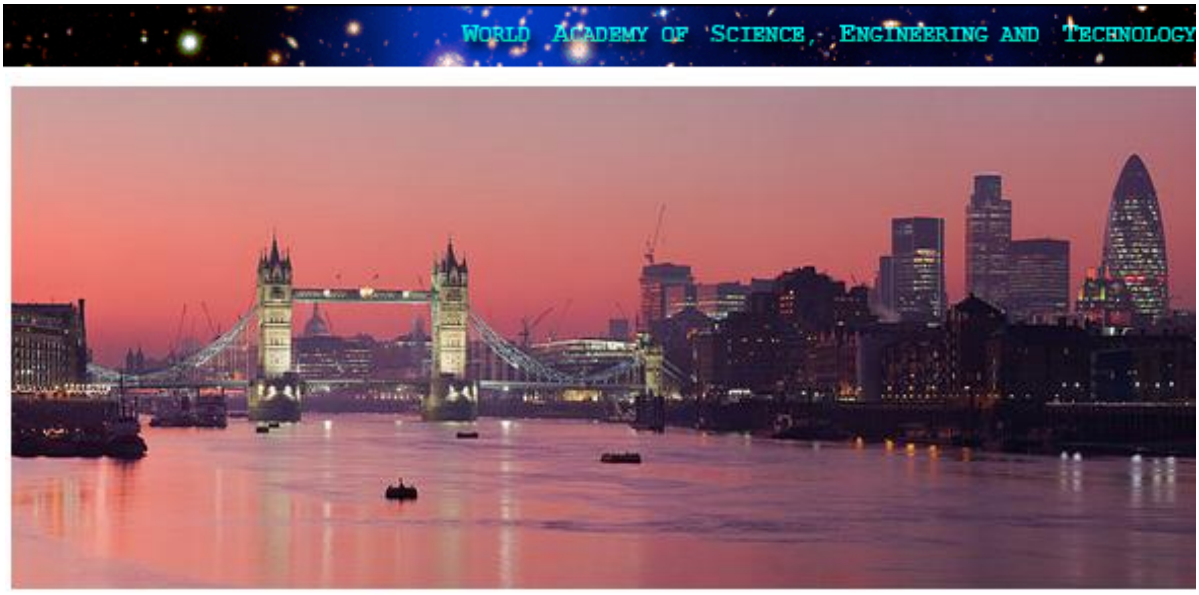

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