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# African Journal of Microbiology Research

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*Full Length Research Paper*

## **Isolation of a *Lactobacillus* strain from aguamiel and preliminary characterization of its antimicrobial components**

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The aim of the study was to characterize the antimicrobial components of *Lactobacillus paracasei* KSI. In the study, a *L. paracasei* KSI strain was isolated and identified from aguamiel using a 16S rRNA, hsp, recA and rpoB genes sequencing. The antimicrobial capacity of the *L. paracasei* strain KSI was determined by agar double layer diffusion technique, while the antagonistic activity of the cell-free extract (E-KSI) was evaluated by agar well diffusion method against different bacterial strains; it demonstrated a wide spectrum of inhibition. Likewise, E-KSI showed stability at different temperatures and digestive enzymes; its activity was lost at pH>5. Subsequently, E-KSI was concentrated (10x) by evaporation increasing its antagonistic effect. Antagonism tests by thin-layer chromatography (TLC)-bioautography of the E-KSI 10x showed the presence of more than one active substances different from lactic acid, possibly of the bacteriocin type. Some of these substances were recovered by extractions with ethyl ether, ethyl acetate and n-butanol respectively. Finally, we evaluated them using antagonism tests by minimum inhibitory concentration (MIC) and microdilution. *L. paracasei* strain KSI generates substances with antibacterial activity having a wide spectrum of inhibition; it is a promising alternative to future biotechnological applications. The strain of *L. paracasei* KSI as well as the antimicrobial components that it generates has important antagonistic properties, making them an interesting biotechnological alternative to be used as probiotic or a safe functional food.

**Key words:** Lactobacillus, antimicrobials, probiotics, biotechnology, food safety.

### **INTRODUCTION**

The group of lactic acid bacteria (BAL) are microorganisms Gram-positive bacilli, do not form spores,

usually immobile, anaerobic, microaerophilic or air tolerant, catalase, oxidase and benzidine negative, without

cytochromes, nor reduce nitrate to nitrite and produce lactic acid as the only or main product of carbohydrate fermentation (Carr et al., 2002). This group of microorganisms is widely distributed in nature and has been isolated from different foods (Azadnia et al., 2011) (milk and dairy products, wines, fruits, vegetables, fishery products, among others), soil, digestive tract, mouth and vagina of mammalian between other sources (Claesson et al., 2007). The type of metabolism developed by the BAL is primarily used to obtain fermented food and beverages (Chilton et al., 2015). The BAL can also play the role of food biopreservatives and bioprotective cultures (Parada et al. 2007; Benmechene et al., 2013), because in addition to competing for nutrients, they also produce metabolites that inhibit the growth of contaminating or pathogenic microorganisms. Antimicrobial capacity starts with the production of lactic acid that participates in the progressive decrease of the pH (Erdogrul and Erbilir, 2006); during the fermentation process, the BAL can produce other substances capable of inhibiting the growth of competing bacteria, among which low molecular weight molecules have been found, the most common being: acetic acid, propionic acid, hydrogen peroxide, carbon dioxide, diacetyl, acetaldehyde, ethanol (Piard and Desmazeaud, 1991; Lahtinen et al., 2011); peptides with antimicrobial properties called "bacteriocins" (Chen and Hoover, 2003; Dobson et al., 2012; Shaikh et al., 2012; Ribeiro et al., 2013; Zendo, 2013; Drissi et al., 2014; Yang et al., 2014) biosurfactant compounds (Rodrigues et al., 2006; Saharan et al., 2011; Sharma and Saharan, 2014) and other compounds not yet characterized. The BAL have the category of the generally recognized as safe (GRAS) granted by the food and drug administration (FDA) of the USA (Zacharof and Lovitt, 2012), and due that the substances that produce during their metabolic processes present activity against various pathogens included bacteria, parasites, fungi and yeasts (Papagianni, 2003; Joerger, 2003; Motta and Brandelli, 2008).

The classification of the BAL was established by Orla-Jensen (1920), and was based on their morphological, metabolic and physiological characteristics. Nowadays they are classified according to the subunit 16S rRNA gene (Olsen et al., 1994; Ben-Amor et al., 2007); its characteristics possess a guanine-cytosine content (GC) less than 50%. This group comprises of microorganisms of the following genera: *Urinae*, *Alloicoccus*, *Carnobacterium*, *Dolosigranulun*, *Enterococcus*, *Globicatella*, *Lactobacillus*, *Lactococcus*, *Lactosphaera*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*,

*Tetragenococcus*, *Vagococcus* and *Weissella* (Cintas et al., 2001; Rojas and Vargas 2008), being *Lactobacillus* the most studied genus, and some members of this genus are considered probiotics (Axelsson, 2004). The use of probiotics is given, by its influence on the intestinal microbiota and its antagonism with the pathogenic bacteria. Recent reports describe the properties of strains of *Lactobacillus* isolated from different environments: *Lactobacillus rhamnosus* isolated from human vagina (Turovskiy et al., 2009), *Lactobacillus plantarum*, *Lactobacillus pentosus* and *Lactobacillus helveticus* isolated from silage (Giraldo et al., 2010), *L. paracasei* isolated from fecal matter of infants (Bendjedou et al., 2012), *Lactobacillus sakei* D98 isolated from sake (Sawa et al., 2013), *L. plantarum* from corn silage (Amortegui et al., 2014), *L. plantarum* isolated from grapes (Chen et al., 2014), and *L. paracasei* SD1 isolated from oral cavity (Wannun et al., 2014), *Lactobacillus casei* MRTL3 all with the ability to produce substances with antagonistic properties against various pathogenic bacteria (Sharma and Saharan, 2014).

Multiple agave species grown in the semi-desert areas of Mexico, such as maguey-pulquero (*Agave atrovirens*), aguamiel is the agave sap, which is collected when the maguey is mature and is used for the production of pulque (a drink with cultural importance in Mexico) contains fructooligosaccharides that are susceptible to fermentation in the colon by colonic microorganisms; it has also been shown to have important prebiotic properties (Romero-López et al., 2015). The objective of this work was the preliminary characterization of inhibitors produced by *L. paracasei* strain KSI isolated from aguamiel, and the evaluation of its antimicrobial activity.

## MATERIALS AND METHODS

### Bacterial identification

The *L. paracasei* strain KSI was isolated from an "agua miel" sample and cultivated in MRS agar and incubate for 48 h at 37°C. The macroscopic characteristics of colonies were evaluated, in addition, the gram staining and catalase and oxidase test were performed (which corresponds to the characteristics of the BAL). The identification of *L. paracasei* KSI was performed by the polymerase chain reaction (PCR) using the 16S rRNA subunit, *hsp*, *recA* and *rpoB* genes. Universal primers were used for the amplification of 16S rRNA gene; E9F: 5'-GAGTTTGATCCTGGCTCAG-3' and E939R: 5'-CTTGTGCGGGCCCCCGTCAATTC-3'; (Forney et al., 2004) and for the amplification of the genes *hsp*, *recA* y *rpoB* were designed primers *hsp*-F: 5'-TGAATCGTCGTAATAATGAGTTG-3' and *hsp*-R 5'-TTTCAATGTTGTGACCAGAC-3'; *recA*-F: 5'-

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GACATCGTGGTTTCAACC-3' and recA-R: 5'-TATGGTCCTGAAAGTACC-3' and rpoB-F: 5'-GATCTTCCCAGATGATGAC-3' and rpoB-R: 5'-TTGAAAGTATGCAAATCGGC-3', respectively. The PCR reactions were performed to a final volume of 50  $\mu\text{L}$ , with 25  $\mu\text{L}$  of PCR Master Mix (2X, Thermo scientific), 1  $\mu\text{L}$  of E9F (25 pmol  $\mu\text{L}^{-1}$ ), 1  $\mu\text{L}$  of E939R (25 pmol  $\mu\text{L}^{-1}$ ), 23  $\mu\text{L}$  of nuclease-free water and 1 bacterial colony as a DNA template. The amplification protocol consisted of an initial denaturation: 94°C/2 min; 35 amplification cycles with denaturation: 94°C/30 s; alignment: 59.8°C/30 s; extension: 72°C/1.30 min and a final extension: 72°C/10 min. PCR products were visualized in a 1% agarose gel stained with ethidium bromide and subsequently purified from agarose gel using the purification system Zymoclean™ Gel DNA Recovery Kit (Zymo Research) following the manufacturer's specifications; finally purified products were sent to the Molecular Biology unit of the Institute of Cellular Physiology of the UNAM for sequencing.

#### Evaluation of the inhibitory activity of *L. paracasei* KSI by agar double layer diffusion technique

*L. paracasei* KSI was cultured in MRS broth at 37°C / 48 h / 150 rpm, after the incubation time, 1  $\mu\text{L}$  of the culture ( $3 \times 10^{10}$  cells) was placed in a well of 1 mm diameter, previously generated with a sterile capillary tube, in MRS and APT agar plates. The agar plates were incubated at 37°C for 48 h and later the bacteria were removed. Finally, the plates were covered with 10 mL of soft agar Müller Hinton, previously inoculated with 1 mL of the indicator strain: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 25923 respectively, all of them adjusted to the concentration of 0.5 of the Mc Farland standard (CLSI, 2017). The plates were solidified in sterile conditions and incubated at 37°C / 24 h for the interpretation.

#### Obtaining cell-free extract of *L. paracasei* KSI and test of antagonism by agar well diffusion assay

*L. paracasei* KSI was cultivated in 100 mL MRS broth and incubated at 37°C/ 48 h/ 150 rpm. After incubation, the culture was centrifuged at 3396 g/ 4°C/30 min, the cell-free extract (E-KSI) was recovered and sterilized by ultrafiltration with 0.22  $\mu\text{m}$  membranes. The pH value was determined to carry out the tests of antagonism by diffusion in agar to be evaluated against the indicator strains, which were cultivated on AST agar plates at 37°C /24 h, to prepare an inoculum adjusted to the 0.5 of Mc Farland standard. The inoculum was cultivated on Müller Hinton agar plates and glass cylinders were placed to generate wells in which 150  $\mu\text{L}$  of E-KSI (0.63  $\mu\text{g}/\mu\text{L}$  of total protein) which was subjected to different conditions were deposited, subsequently the plates were incubated at 37°C / 24 h to observe areas of inhibition (Valgas et al., 2007).

#### Evaluation of the stability of the cell-free extract (E-KSI)

To determine the stability of the cell-free extract (E-KSI), different treatments were performed. Thermal stability was evaluated at 121°C/15 min; 100°C/30 min; 37, 25, 4, -10 and -80°C for a week, respectively. For pH stability, different pH conditions were adjusted to pH = 5 with acetate buffer (0.1 mol  $\text{L}^{-1}$ ), pH= 6.8 with Tris (0.1 mol  $\text{L}^{-1}$ ), pH=7.4 with PBS (0.1 mol  $\text{L}^{-1}$ ) and pH=8 with Tris (0.1 mol  $\text{L}^{-1}$ ). Finally the stability of the E-KSI was evaluated with the digestive enzymes: proteinase K, lysozyme, trypsin and chymotrypsin, which were used at a final concentration of 1 mg  $\text{mL}^{-1}$ . Samples were incubated at 37°C for 24 h, after incubation time

each of the enzymes was inactivated at 100°C for 15 min. Each one of the treated samples was evaluated by test of antagonism by agar well diffusion assay.

#### Evaluation of the E-KSI (10x) through antagonism tests by thin-layer chromatography (TLC)–bioautography

For obtaining of E-KSI (10x), the strain of *L. paracasei* KSI was incubated in 100 mL of MRS broth at 37°C/48 h/150 rpm recovering the supernatant by centrifugation, which was evaporated at 65°C at reduced pressure, until the almost dry product was obtained, the evaporated solvent was also recovered. The concentrated product was rehydrated to a final volume of 10 mL (10x) with sterile distilled and deionized water, while the recovered solvent did not receive any subsequent treatment. All the products were sterilized by filtration with 0.22  $\mu\text{m}$  membranes to evaluate antagonistic activity. For analysis of E-KSI (10x) a thin layer chromatography (TLC) using a stationary phase of plates silica gel ( $\text{SiO}_2$ ) was performed. Samples were analyzed and compared with lactic acid using a mobile phase of a mixture of ethyl acetate: hexane (9:1) and methanol: acetic acid (9:1), and the developed was performed by UV light, iodine and ninhydrin, respectively. While for bioautography (Mehrabani et al., 2013), silice plates were placed at 65°C for eluents evaporation. Subsequently, the chromatographic plates were placed on Müller Hinton agar plates (modified from Mehrabani). Finally, the plates were covered with 10 mL of soft agar Müller Hinton, previously inoculated with 1 mL of the indicator strain, adjusted to the concentration of 0.5 of the Mc Farland standard. The plaques were cultivated and incubated at 37°C/24 h for interpretation.

#### Obtaining antimicrobial components from E-KSI (10x) by extraction with different organic solvents

Aliquots of E-KSI (10x) were mixed with equal volumes (1:1) of isopropyl alcohol, butyl alcohol, ethyl acetate, hexane, chloroform and ethyl ether, respectively. Subsequently the samples were centrifuged and organic fractions were separated and evaporated to obtain the dry product which was weighed and reconstituted in sterile deionized water. The residual aqueous phase was reconstituted and both products were tested by agar well diffusion assay.

#### E-KSI 10x active fractions evaluation by minimum inhibitory concentration (MIC)

For MIC test (Balouiri et al., 2016), a 96 well microplate and aliquots of E-KSI (10x) extracted with ethyl ether, ethyl acetate and butyl alcohol was used. Serial dilutions were made with each E-KSI (10x) extraction. *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 25923 cultures adjusted to 0.5 of Mc Farland standard were added and finally incubated at 37°C/24 h for interpretation.

## RESULTS

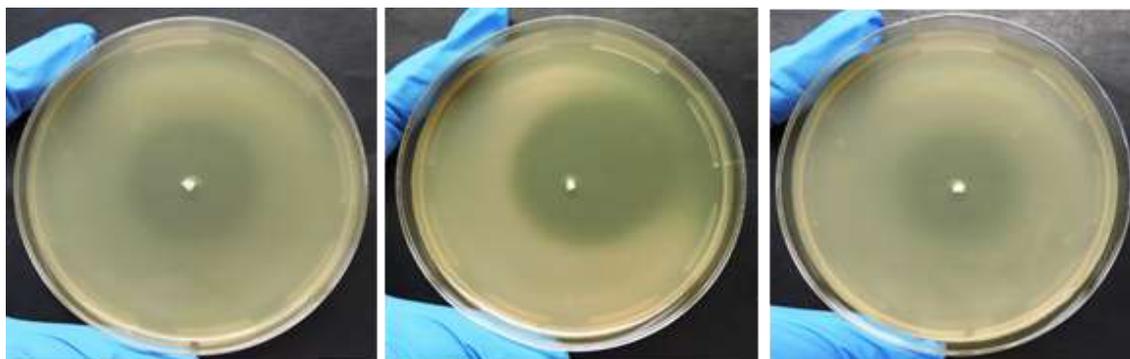
### Isolation and identification of *L. paracasei* KSI

After 48 h incubation in MRS agar there were isolated in beige colony with regular edges, convex, with creamy consistency, negative to catalase and oxidase, and microscopically observed as Gram positive bacilli. The

**Table 1.** Evaluation of the antagonistic capacity of *L. paracasei* KSI.

Indicator strain	Agar double layer diffusion technique	Agar well diffusion assay			
		E-KSI	MRS broth pH= 4	E-KSI (10X)	Solvents
		Inhibition halo (mm)			
<i>E. coli</i> ATCC 25922	34.0	13	-	22	-
<i>P. aeruginosa</i> ATCC 27853	35.0	13	-	21	-
<i>S. aureus</i> ATCC 25923	32.0	-	-	21	-

The assays shown were performed in triplicate, obtaining the same results for each test.



**Figure 1.** Evaluation of the inhibitory activity of *L. paracasei* KSI by agar double layer diffusion technique. From left to right, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 y *S. aureus* ATCC 25923.

molecular identification was performed by PCR amplification and sequencing of 16S rRNA (957 bp), *hsp* (452 bp), *recA* (578 bp) and *rpoB* (3495) genes. The amplicons were sequenced in both directions and the sequences were compared in the GenBank database for homology search using the Basic Local Alignment search Tool (Blastn). The 16S ribosomal subunit gene showed a 100% of identity with strains of *L. rhamnosus*, *L. casei* and *L. paracasei*, respectively, while the *hsp*, *recA* and *rpoB* genes showed a 100% of identity and similarity with different strains of *L. paracasei*. Thus the isolated strain was called *L. paracasei* KSI.

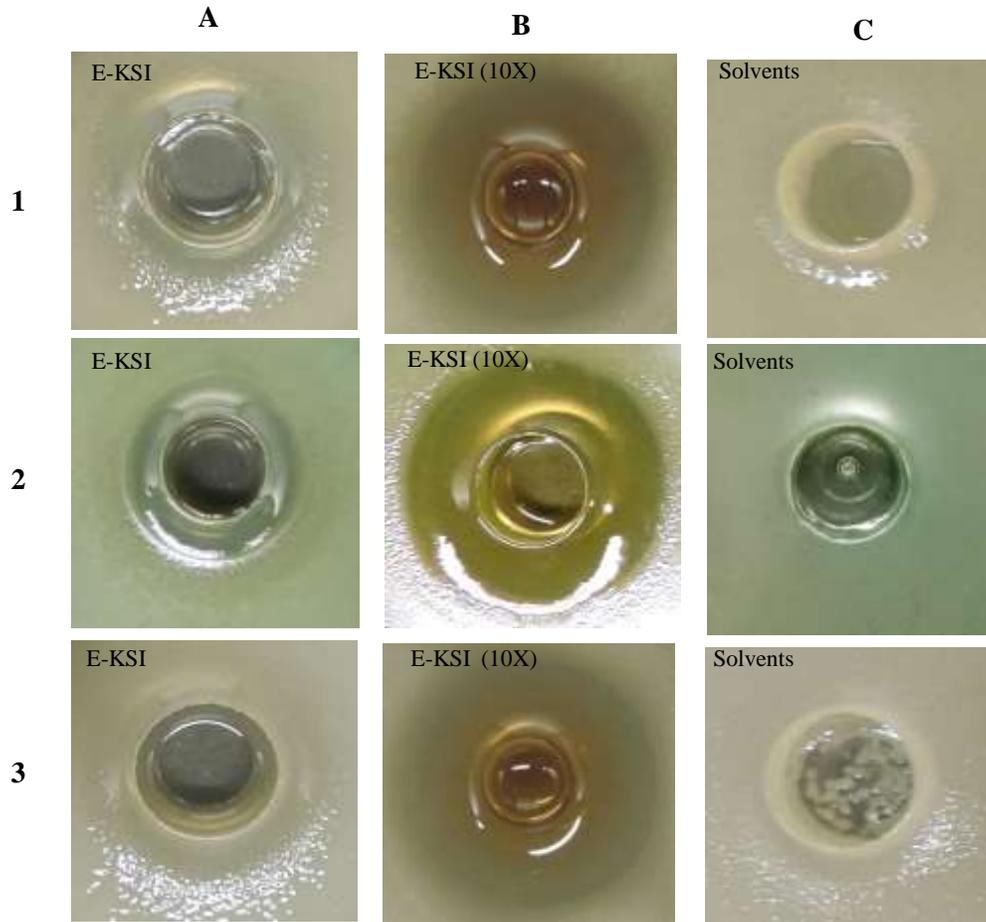
#### Evaluation of the antagonistic capacity of *L. paracasei* KSI

The evaluation of the antagonistic capacity of the strain of *L. paracasei* KSI was evaluated in a solid medium by agar double layer diffusion technique, showing an important inhibitory effect against *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 y *S. aureus* ATCC 25923 strains on MRS agar (Table 1, Figure 1), but not on APT agar where inhibition halos were not observed. To evaluate the antagonistic capacity generated by the strain in liquid medium, we used the test of antagonism by agar well diffusion assay and cell-free extract (E-KSI)

recovered at 48 h with pH 4 and MRS broth at pH 4 as control. The results show that there is a lower inhibitory effect in the test of antagonism by agar well diffusion assay compared to the agar double layer diffusion technique against *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 strains, but no inhibitory effect against *S. aureus* ATCC 25923 strain, it was also observed that the pH did not participate in the antagonist effect (Table 1, Figure 2A). However, when the E-KSI (10x) was evaluated, it was observed that an increase in inhibitory effect against the three indicator strains (Table 1, Figure 2B), without the solvents present in the culture medium participated in this effect (Table 1, Figure 2C).

#### Stability evaluation of E-KSI in different conditions of temperature, pH and digestive enzymes

After evaluating the inhibitory action of E-KSI extract in a liquid medium its stability was determined at different temperatures (121, 100, 37, 25, 4, -10 and -80°C) and pH values (pH = 5, pH= 6.8, pH=7.4 and pH=8), as well as its sensitivity to digestive enzymes (proteinase K, lysozyme, trypsin and chymotrypsin, respectively). After each treatment, diffusion tests were performed and compared with *E. coli* ATCC 25922, *P. aeruginosa* 27853 and *S. aureus* 25922, respectively. The results show the



**Figure 2.** Test of antagonism by agar well diffusion assay of E-KSI. Rows, 1: *E. coli* ATCC 25922, 2: *P. aeruginosa* ATCC 27853 and 3: *S. aureus* ATCC 25923. Columns, A: E-KSI, B: E-KSI (10X) and C: Solvents.

thermal stability of E-KSI in the temperature ranges evaluated, however, the activity is lost at pH values higher than 5. Finally, the extract maintains its antagonistic effect after being treated with enzymatic digestion (Table 2).

#### Evaluation of E-KSI (10X) by thin-layer chromatography (TLC)–bioautography and MIC

For antagonism test bioautography, firstly it compared the migration profile of E-KSI (10x) with the lactic acid profile (8.5%) using thin-layer chromatography. For thin-layer chromatography we used two elution systems, one with less polar characteristics (ethyl acetate: hexane (9:1)) and another with more polar characteristics (methanol: acetic acid (9:1)), observing that the E-KSI (10x) has a migration profile different from lactic acid and that E-KSI (10x) presents a diversity of compounds that was separated by this technique. Similarly, when it observed

the sample of E-KSI (10x) eluted with the methanol: acetic acid mixture, the presence of polar molecules that react when revealed with ninhydrin was demonstrated, which suggested protein structures or biogenic amines (Figure 3). The bioautography assay showed an inhibition area along the migration profile of E-KSI (10X) different from lactic acid profile, indicating more than one component of the extract promotes an inhibitory effect (Figure 4). From E-KSI (10x) there obtained the active fractions of the organic extractions made with n-butanol (But), ethyl acetate (EA) and ethyl ether (EE) but not with hexane and chloroform (Table 3 and Figure 4), which were evaluated first by antagonism tests by agar well diffusion assay method (Figure 5) and later by MIC.

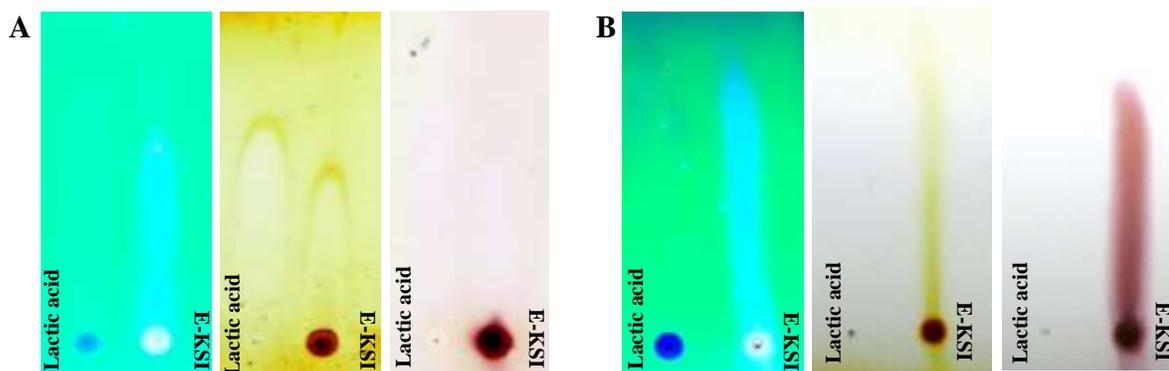
#### Minimum inhibitory concentration (MIC)

For the MIC test the extractions obtained with the organic solvents were used. The stock solutions obtained were

**Table 2.** Evaluation of E-KSI submitted to different treatments by agar diffusion assay.

Treatment on the extract KSI (E-KSI)	Inhibitory activity (mm)		
	<i>E. coli</i> ATCC 25922	<i>P. aeruginosa</i> ATCC 27853	<i>S. aureus</i> ATCC 25923
<b>Temperature</b>			
-80°C	13.0	13.0	-
-10°C	13.0	13.0	-
4°C	13.0	13.0	-
25°C	13.0	13.0	-
37°C	13.0	13.0	-
100°C	13.0	13.0	-
121°C	13.0	13.0	-
<b>Enzymes digest</b>			
Trypsin	13.0	13.0	-
Chymotrypsin	13.0	13.0	-
Proteinase K	13.0	13.0	-
Lisozime	13.0	13.0	-
<b>pH</b>			
4.0	13.0	13.0	-
5.0	-	-	-
6.8	-	-	-
7.4	-	-	-
8.8	-	-	-

The assays shown were performed in triplicate, obtaining the same results for each test.

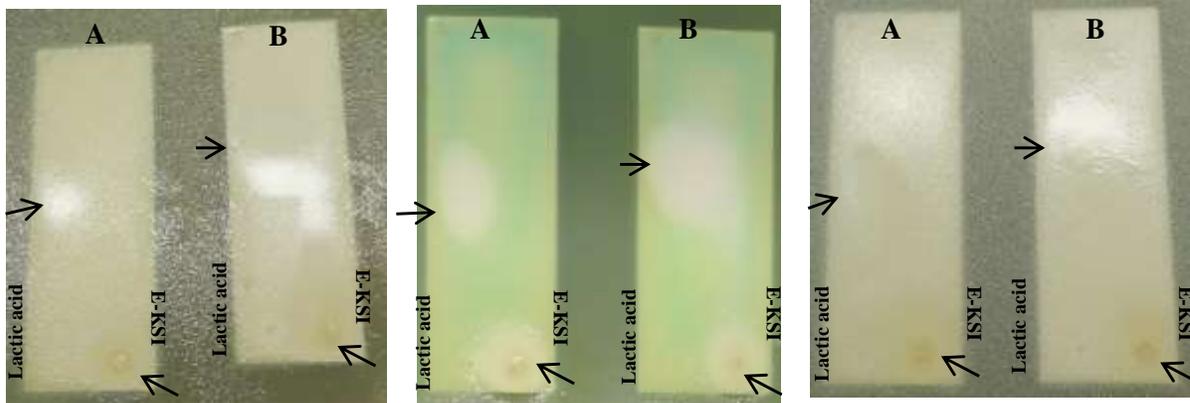


**Figure 3.** Analysis of E-A5 (10X) using thin-layer chromatography (TLC). A. TLC using as eluent ethyl acetate: hexane (9:1). B. TLC using as eluent methanol: acetic acid (9:1); revealed from left to right with UV, iodine and ninhydrin, respectively.

14 mg 500  $\mu\text{L}^{-1}$  from ethyl ether extraction, 34 mg 500  $\mu\text{L}^{-1}$  from the ethyl acetate and 122 mg 500  $\mu\text{L}^{-1}$  from n-butanol. The MIC of recovered fraction with ethyl ether was 350  $\mu\text{g}$  for *E. coli* and *S. aureus*, respectively, whereas for *P. aeruginosa* was 700  $\mu\text{g}$ . Besides, the MIC from recovered fraction of ethyl acetate was 850  $\mu\text{g}$  for *E. coli* and 425  $\mu\text{g}$  for *S. aureus* and *P. aeruginosa*, respectively. Finally the MIC for the fraction obtained from n-butanol was 1525  $\mu\text{g}$  for three strains.

## DISCUSSION

Currently, the antimicrobial capacity of bacteria with probiotic potential is still being explored, one of the most studied genera is *Lactobacillus*. Today the identification of these microorganisms is based on their metabolic characteristics and sequencing of the 16S rRNA subunit, it also demonstrate its low phylogenetic to differentiate the species level and poor discriminatory power for some



**Figure 4.** Analysis of E-KSI (10X) through essays of autobiography. From left to right, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 25923. A: TLC eluted with ethyl acetate: hexane (9:1). B: TLC eluted with methanol: acetic acid (9:1), the arrows indicate the halos of inhibition present. Eluted samples with methanol: acetic acid has inhibition along the bleed.

**Table 3.** Evaluation of E-KSI (10x) submitted to extractions with organic solvents by agar diffusion assay method.

Treatment on the extract E-KSI	Inhibitory activity (mm)		
	<i>E. coli</i> ATCC 25922	<i>P. aeruginosa</i> ATCC 27853	<i>S. aureus</i> ATCC 25923
<b>Extraction of E-KSI 10x (organic phase)</b>			
MRS control (-)	-	-	-
n-Butanol (But)	18.0	20.0	18.0
Ethyl acetate (EA)	18.0	20.0	18.0
Hexane (Hex)	-	-	-
Chloroform (CF)	-	-	-
Ethyl ether (EE)	12.0	14.0	14.0
<b>Extraction of E-KSI 10x (organic aqueous)</b>			
E-KSI 10x control (+)	22.0	21.0	22.0
n-Butanol (But)	15.0	14.0	-
Ethyl acetate (EA)	16.0	16.0	15.0
Hexane (Hex)	16.0	18.0	18.0
Chloroform (CF)	16.0	17.0	18.0
Ethyl ether (EE)	17.0	17.0	16.0

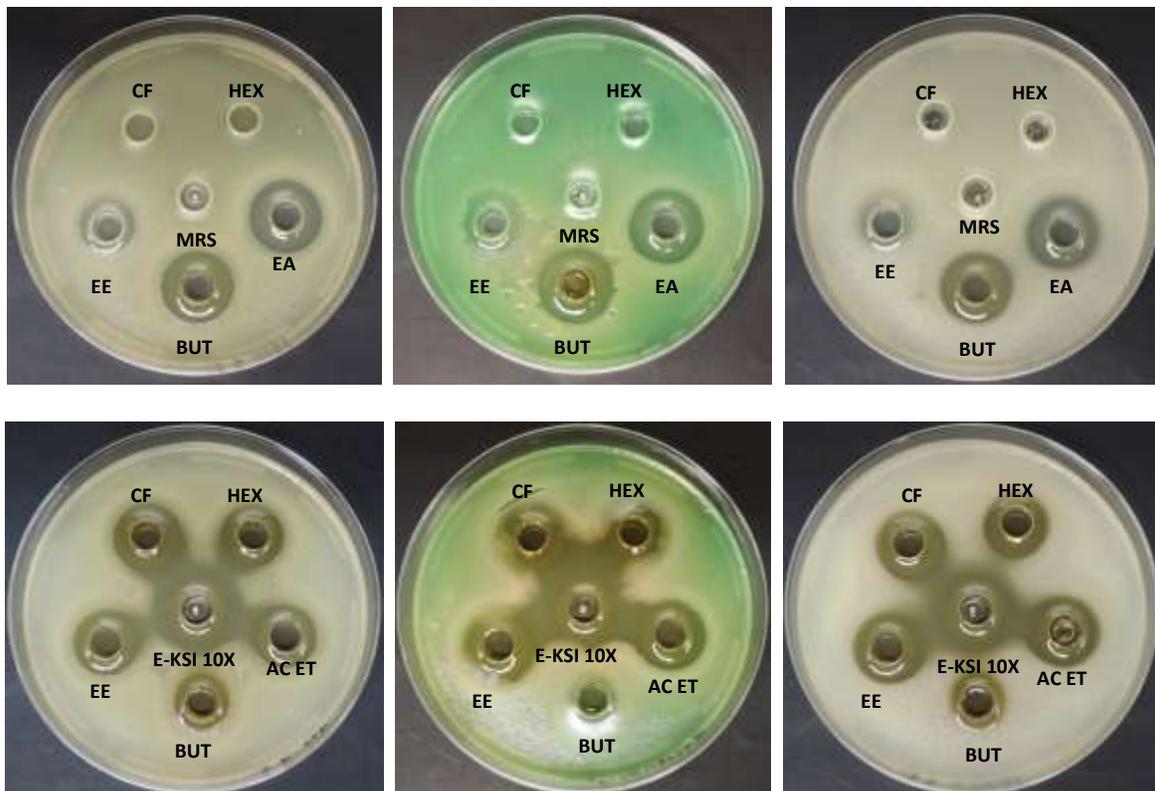
The assays shown were performed in triplicate, obtaining the same results for each test.

genera (Janda and Sharon, 2007). So it was necessary to analyze diverse housekeeping genes (*hsp*, *recA* and *rpoB*), which contribute to a more efficient bacterial identification at the level of genus, species and subspecies (Bou et al., 2011).

On the other hand, the importance of these bacteria has lately focused on their ability to generate antagonistic effect or produce substances for this purpose against competing bacteria, mainly pathogenic. In this study the ability of the *L. paracasei* KSI strain to generate antibacterial substances in both solid and liquid media was demonstrated. However, there was difficulty in

recovering the antimicrobial substances from the solid medium in comparison with the liquid medium when extractions with organic solvents were related (data not shown).

Studies for the production of antimicrobial substances generally focus on *in vitro* tests with the use of complex media designed for the proliferation of BAL (Garsa et al., 2014), as medium MRS. In this study, the effectiveness of APT agar (designed for the efficient proliferation of *Lactobacillus*) was evaluated for this same purpose, without obtaining positive results. This result verifies that the components of the culture medium are important for



**Figure 5.** Antagonism tests of E-KSI extractions with different organic solvents. From left to right, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 25923. Plates from the top: Extractions of E-KSI 10X (organic phase). Plates from the bottom: Extractions of E-KSI 10X (organic aqueous).

the production of antimicrobial substances, in such a way that for their generation *in vivo*, the nutritional conditions suitable for the microorganism are required (Klaenhammer et al., 2007).

The ability of BALs to secrete bacteriocin-like protein substances in liquid culture media is widely demonstrated (Bodaszewska-Lubas et al., 2012). The study of protein characteristics of these substances was demonstrated by evaluation of stability at different temperatures, pH and digestive enzymes sensitivity. This study showed that *L. paracasei* KSI strain produced stable thermo-substances in liquid medium results was similar to Chen et al. (2014) who evaluated the thermal stability of the Plantaricin Y bacteriocin (isolated from a strain of *L. plantarum*), which were resistant to the activity of the digestive enzymes with which they were treated. On the other hand, in the results obtained by Wannun et al. (2014), the cell-free extract obtained from *L. paracasei* SD1 lost activity when treated with proteinase K and lysozyme.

When evaluating the stability of the pH changes, we observed that antagonistic activity is lost when the value exceeds a pH = 5, different from that observed by Bendjeddou et al. (2012), which evaluated the activity of Paracaseicin A bacteriocin of strain BMK2005 of *L.*

*paracasei*, losing activity at pH = 7 and decreasing its inhibitory capacity with the action of proteolytic enzymes.

However, it is observed that adjusting E-KSI to a pH = 4 value recovers its inhibitory activity, which reveals the cationic characteristics of the substances it contains. When the extract was concentrated by evaporation, an increase in the inhibitory effect was observed. On the other hand, the participation of lactic acid in the antagonist effect was analyzed since this is one of the main metabolites generated by the BAL and it was demonstrated that high concentrations of this are required to present the same inhibitory effect as the extract.

From the E-KSI (10X), active fractions were recovered by extraction with ethyl ether, ethyl acetate and n-butanol, which implies that E-KSI contains molecules with different polarities (possibly of the biosurfactant type), as observed in thin-layer chromatography and the bioautography tests. All recovered fractions were evaluated by agar diffusion antagonism assays, in the same way the residual aqueous phase generated from each extraction was evaluated and also showed an antagonistic effect when it was evaluated. The results obtained were similar to that reported by Shiba et al.

(2013) that was able to recover active fractions of bacteriocins type from extractions made with n-butanol from cell-free extracts obtained from cultures of strain *L. brevis* FPTLB3, however, it did not recover active fractions with non-polar solvents. The above demonstrates the presence of more than one active molecule different from lactic acid, possibly of the bacteriocins type, as suggested by the ninhydrin reaction observed in TLC. Finally, it was determined that molecules extracted with ethyl ether required lower concentration when evaluated by minimum inhibitory concentration (MIC) test.

In conclusion the strain of *L. paracasei* KSI is capable of generating several molecules with antagonistic activity against *E. coli* ATCC25922, *P. aeruginosa* ATCC27853, and *S. aureus* ATCC 25923, in both liquid and solid media. Therefore the results presented in this study offers a promising alternative as probiotic potential.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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*Full Length Research Paper*

# **Internal transcribed spacers (ITS) based identification of *Trichoderma* isolates and biocontrol activity against *Macrophomina phaseolina*, *Aspergillus niger* and *Meloidogyne incognita***

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Ten *Trichoderma* isolates were isolated from different locations in Egypt. Amplification and sequencing of internal transcribed spacers (ITS) was employed to identify *Trichoderma* isolates that exhibited from 99 to 100% identity with three species of *Trichoderma*: *Trichoderma harzianum*, *Trichoderma asperellum* and *Trichoderma longibrachiatum*. The biocontrol activity of *Trichoderma* isolates against *Macrophomina phaseolina*, *Aspergillus niger* and *Meloidogyne incognita* was tested *in vitro* and under greenhouse conditions. The results show that the isolate Th2 (*T. harzianum*) gave the best antagonism against *M. phaseolina* and *A. niger* with inhibition rates of 72.85 and 64.28%, respectively. Moreover, the isolate Ta1 (*T. asperellum*) was the best efficient isolate in reduction of each second stage juveniles (J2), number of galls, egg masses and females per root system with 90.33, 90.59, 90.06 and 85.50%, respectively. Treatment with *Trichoderma* isolates improved tomato growth parameters (root length, plant height, roots and shoots fresh weight and shoots dry weight).

**Key words:** *Trichoderma*, internal transcribed spacers (ITS), antagonism, *Macrophomina phaseolina*, *Aspergillus niger*, *Meloidogyne incognita*.

## **INTRODUCTION**

The chemical pesticides are the first choice for farmers to control plant pathogens in order to obtain abundant crop yield (Junaid et al., 2013). It was estimated that 12% of crop loss is due to plant pathogens (Sharma et al., 2012). The long term uses of pesticides can cause severe environmental and health problems in addition to their expensive costs for developing countries. *Trichoderma* is

a famous saprophytic fungus that can be isolated from any soil and can be used as efficient biocontrol agent due to its high ability to reproduce and colonize (Pandya et al., 2011; Pal and Gardener, 2006; El-Hassan et al., 2013; Rao et al., 2015). *Trichoderma* fight against many fungal phytopathogens such as *Phythium*, *Phytophthora*, *Macrophomina*, *Aspergillus*, *Rhizoctonia* and *Fusarium*

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**Table 1.** Isolation sites and identification of *Trichoderma* isolates based on ITS data

Isolate code	Source	BLASTn results identity (%)	<i>Trichoderma</i> spp.
Ta1	Qalubia governorate	99	<i>Trichoderma asperellum</i>
Ta2	Gharbia governorate	100	<i>Trichoderma asperellum</i>
Ta3	Behira governorate	100	<i>Trichoderma asperellum</i>
Ta4	Gharbia governorate	99	<i>Trichoderma asperellum</i>
Th1	Dakhalia governorate	100	<i>Trichoderma harzianum</i>
Th2	Ismailia governorate	99	<i>Trichoderma harzianum</i>
Th3	Ismailia governorate	99	<i>Trichoderma harzianum</i>
Th4	Sharkia governorate	99	<i>Trichoderma harzianum</i>
Th5	Agricultural Research Center, Giza, Egypt	99	<i>Trichoderma harzianum</i>
TI	Agricultural Research Center, Giza, Egypt	99	<i>Trichoderma longibrachiatum</i>

using many biocontrol weapons as mycoparasitism, production of antibiotics and competition of pathogens for food and space (Benítez et al., 2004; Rayatpanah et al., 2011; Krishnamurthy and Shashikala, 2006). Recently, the global attention rely on *Trichoderma* as safe alternative of pesticides and this led to increase in the number of *Trichoderma* biocontrol products (Woo et al., 2014).

*Macrophomina phaseolina* and *Aspergillus niger* are considered destructive pathogens of many important and economically crops causing huge loses of each quantity and quality of crop yield (Khaledi and Taheri, 2016; Njoki et al., 2017; Bhale et al., 2013). *M. phaseolina* is a dangerous fungus that can infect many of important crops as corn, cotton, sesame, soy bean and sunflower causing diseases such as soy bean charcoal rot (Khaledi and Taheri, 2016; Aly et al., 2007). In addition, *A. niger* is a common saprophytic fungus that can cause dangerous diseases as black-rot and collar rot in crops like onion and peanut leading to yield loss from 5 to 40% (Khokhar et al., 2012; Gajera et al., 2011; Bhale et al., 2013). Moreover, root knot nematode (*Meloidogyne incognita*) is one of virulent parasites that attack many crops and can lead to destructive damages in crop yield range from 40 to 50% (Bakr et al., 2011; Katooli et al., 2010; Sikora and Fernandez, 2005; Karssen and Moens, 2006; Maqbool and Kerry, 1997). The previous studies indicated a vital role for *Trichoderma* species in biocontrol of *M. phaseolina*, *Aspergillus* species and *M. incognita* (Al-Hazmi and Javeed, 2016; Khaledi and Taheri, 2016; Mendoza et al., 2015; Shoib et al., 2018; Gajera et al., 2011; Sharon et al., 2011). As the cell wall of nematodes and pathogenic fungi is mainly composed of chitin, *Trichoderma* have chitinase enzymes able to degrade the cell wall of these pathogens (Loc et al., 2011; Haran et al., 1995; Ike et al., 2006). Identification of *Trichoderma* spp. to be applied in the field of biological control is an important issue. It was reported that identification of *Trichoderma* based on morphological characters can give misleading results (Fahmi et al., 2016). Recently, molecular identification based on internal transcribed

spacers (ITS) amplification and sequencing is common and highly trusted (Savitha and Sriam, 2015; Fahmi et al., 2016; Oskiera et al., 2015; Jiang et al., 2016).

In this study, 10 isolates of *Trichoderma* spp. were isolated from soil rhizosphere of different locations in Egypt, characterized on molecular level and screened for their antagonistic ability against *M. phaseolina*, *A. niger* and root knot nematode.

## MATERIALS AND METHODS

### Isolation of *Trichoderma* isolates

Ten (10) isolates of *Trichoderma* spp. were isolated from soil rhizosphere of different sites in Egypt (Table 1) cultivated with different crops as described by Fahmi et al. (2016). Several soil samples were obtained from a 15 cm depth, placed in sterile bags and transferred to the laboratory for isolation process. Serial dilutions were made and 250 µL of 10<sup>3</sup> dilution was distributed onto petri dishes containing potato dextrose agar medium. The plates were then incubated at 28°C for one week for isolation of single colonies.

### Soil borne pathogens

Isolates of *M. phaseolina* and *A. niger* were kindly provided by Faculty of Science, Zagazig University, Egypt.

### DNA extraction from *Trichoderma* isolates

DNA isolation of *Trichoderma* was performed as described by Al-Samarrai and Schmid (2000). The clear sharp bands were indicator for the quality of DNA.

### Molecular identification of *Trichoderma* isolates

Polymerase chain reaction (PCR) was utilized to amplify the internal transcribed spacer regions of *Trichoderma* using ITS1 (5' - TCC GTA GGT GAA CCT GCG G - 3') and ITS4 (5' - TCC TCC GCT TAT TGA TAT GC - 3') primers. PCR conditions were performed as described by Loc et al. (2011). PCR products were first purified using QIAquick PCR Purification Kit (QIAGEN Cat. No. 28104). Sequencing was performed using Big Dye Terminator v3.1 Cycle

Sequencing Kit in a total volume of 20  $\mu$ L using 3500 Genetic Analyzer, Applied Biosystems (Daejeon, Korea).

### PCR amplification of chitinase gene

Amplification of chitinase gene was done using specific primers: CHI-F (5-ATG TTG GGC TTC CTC GGA-3) and CHI-R (5-TTC GGG ATG GTT GTC ATA CTG-3) as described by Loc et al. (2011).

### Antagonism of *Trichoderma* isolates

The antagonism of *Trichoderma* isolates against *M. phaseolina* and *A. niger* was evaluated in dual culture plate technique by measuring the radial growth inhibition of pathogen(s) as described by Abdel-lateif et al. (2017).

### Multiplication of *M. incognita*

Root-knot nematode, *M. incognita* was identified by observation of perineal patterns according to Hartman and Sasser (1985). Multiplication was done under greenhouse conditions at the experimental greenhouse, Department of Agricultural Botany, Faculty of Agriculture, Menoufia University, Egypt. Susceptible tomato seedlings Cv. GS (*Solanum lycopersicum* Mill) was transplanted in plastic pots 15 cm in diameter filled with sterilized clays and soil (1:2 v/v). Seedlings were then inoculated by one egg mass of *M. incognita*. Agricultural operations were carried as needed.

### Preparation of *M. incognita* inoculum

Two months heavily galled tomato roots previously infested with *M. incognita* were used to prepare nematode inoculum. Massive infested roots removed from the pots and gently washed using tap water to separate the adherent soil particles. Then roots were cut into small pieces and macerated for two periods of 10 s each at high speed by using a blender. The macerated root solution then placed in a jar containing 0.5% of sodium hypochlorite (NaOCl) as described by Hussey and Barker (1973). The Jar was vigorously shaken for 3 min to release the eggs from the egg matrix with the help of NaOCl. The resulting solution then poured through serial sieves to separate the root tissue. Eggs were collected on the last sieve (20  $\mu$ m) and washed several times with tap water to eliminate NaOCl residuals. The eggs were then transferred to a flask containing tap water. The number of eggs/ ml was estimated by counting 4 samples of 1 ml using a counting dish under a stereomicroscope.

### Preparation of *Trichoderma* inoculum

The *Trichoderma* isolates were cultured on potato dextrose agar (PDA) and incubated at 26°C for 10 days. The conidia were collected from the surfaces of plates by flooding with sterile distilled water and gently scraping the colony surface with a sterile scraper (Jansson et al., 1985).

### Greenhouse experiment

Greenhouse experiment was carried out to evaluate the effect of 10 *Trichoderma* isolates on *M. incognita*. Four weeks old tomato seedlings (Cv. GS) were transplanted into plastic pots (15 cm

diameter) containing sterilized sand-clay soil (2:1 v/v). Each plant is inoculated with 3000 eggs of *M. incognita* and *Trichoderma* spore suspension ( $10^8$  g<sup>-1</sup> of soil) around the young hairy roots and were mixed well within the pots. Control treatments included untreated seedlings and nematode treated seedlings without *Trichoderma*. Treatment with vydate (abroad spectrum nematicide) was used for comparison.

The treatments were replicated three times in a completely randomized block design under greenhouse conditions. Tomato plants were observed, watered and fertilized with a nutrient solution according to Epstein (1972).

### Plant growth and nematode parameters

Two months after nematode inoculation, tomato plants were carefully uprooted. The roots were cautiously washed with running tap water. Plant growth parameters as root length (cm), plant height (cm), fresh weight of roots (g), fresh weight of shoots (g) and dry weight of shoots (g) were recorded. Number of galls/root system was counted directly, while number of second stage juveniles (J2S)/250 g soil was evaluated using serial sieves and modified Baermann technique as described by Goody (1957). Egg-masses were stained prior to counting by dipping the infected roots in 0.015% Phloxine-B solution for 20 min as described by Daykin and Hussey (1985). Number of females/root system was determined by cutting the root system to small pieces and submerging the roots in a beaker full of tap water for four days at room temperature until they became soft. Roots pieces were then washed through 500 and 250  $\mu$ m sieves to separate the females from the root debris and counted under a stereomicroscope.

### Data and cluster analysis

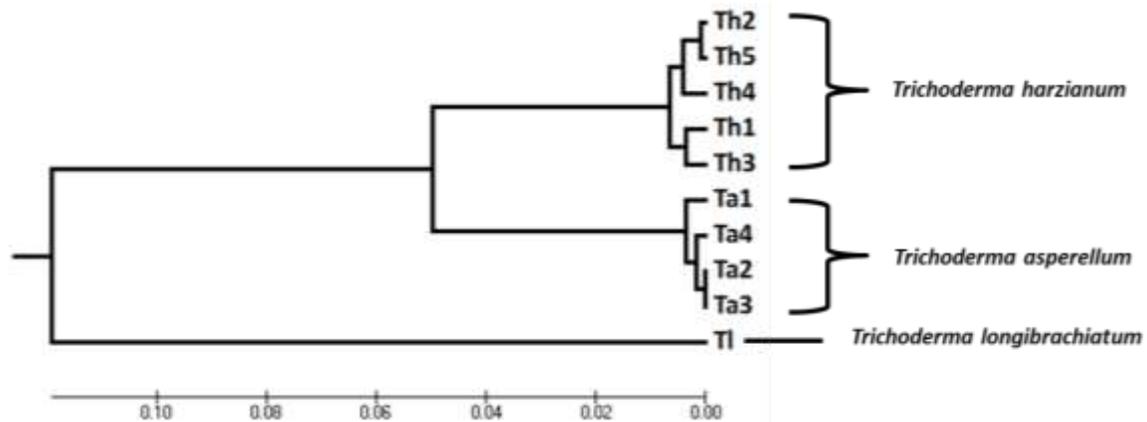
BLASTn was used to compare *Trichoderma* sequencing results with known sequences on NCBI site (<http://www.ncbi.nlm.nih.gov/>). The alignment and phylogenetic analyses were conducted using MEGA version 6 (Tamura et al., 2013). The obtained data of antagonism was analyzed using costat 6.3 version program. Analysis of variance and comparison of means were done at the 5% level of significance according to the Duncan's multiple range test (1955).

## RESULTS AND DISCUSSION

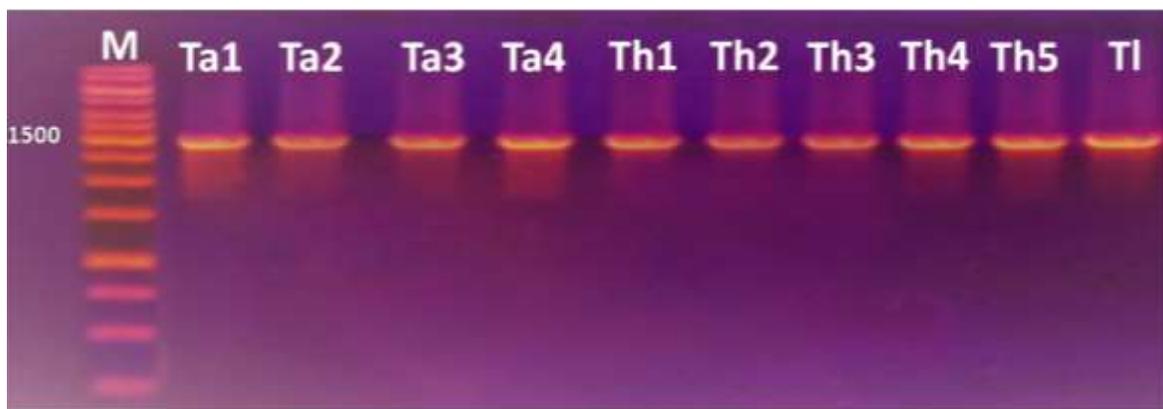
### Isolation and molecular identification of *Trichoderma* isolates

Ten isolates of *Trichoderma* were isolated from rhizosphere soil samples collected from different sites in Egypt (Table 1). PCR based on ITS primers was used to amplify ITS region and gave one band about 600 bp. The PCR products were sequenced and the sequencing data was entered on NCBI site to search BLAST and compare these data with published ITS data. The amplified ITS regions of *Trichoderma* isolates were exhibited from 99 to 100% identity with three species of *Trichoderma*; five isolates were found to belong to *Trichoderma harzianum* (Th1, Th2, Th3 Th4 and Th5), four isolates classified as *Trichoderma asperellum* (Ta1, Ta2, Ta3 and Ta4), while one isolate (TI) was identified as *Trichoderma longibrachiatum* (Table 1).

The phylogeny analysis supported the ITS identification



**Figure 1.** Phylogeny analysis of *Trichoderma* isolates based on ITS data using MEGA 6 (Tamura et al., 2013).



**Figure 2.** Amplification of chitinase gene in *Trichoderma* isolates based CHI-F and CHI-R primers, M; 1 kb plus DNA ladder.

and divided the isolates of *Trichoderma* into three clusters, the first cluster included the isolates of *T. harzianum* (Th1, Th2, Th3, Th4 and Th5). The second cluster included the isolates of *T. asperellum* (Ta1, Ta2, Ta3 and Ta4); finally, the third cluster contained the isolate TI of *T. longibrachiatum* (Figure 1).

These results confirm the efficiency of barcode DNA and are compatible with previous studies that employed ITS to identify *Trichoderma* spp. (Wu et al., 2017; Fahmi et al., 2016; Savitha and Sriram, 2015; Oskiera et al., 2015).

### Chitinase gene of *Trichoderma*

However, chitin is key component of pathogen cell walls, *Trichoderma* secrete highly active chitinases that can destroy these cell walls and feed on them (Seidl-Seiboth et al., 2014; Hassan et al., 2015; Prasetyawan et al., 2018). Chitinase 42 is one of *Trichoderma* endochitinases that can degrade the  $\beta$ -1, 4-glycosidic

bonds between the N-acetyl glucosamine residues of chitin (Hassan et al., 2015). In this study, PCR based specific primers (CHI-F and CHI-R) was utilized to amplify and detect the chitinase 42 gene in *Trichoderma* isolates as shown in Figure 2. The PCR gave one band about 1500 bp homolog to that obtained by Loc et al. (2011) and confirmed the presence of this gene in all tested isolates. The amplification of chitinase gene confirms that these isolates are *Trichoderma* since the primers are specific for *Trichoderma* chitinases.

### Evaluation of *Trichoderma* antagonism against *M. phaseolina* and *A. niger*

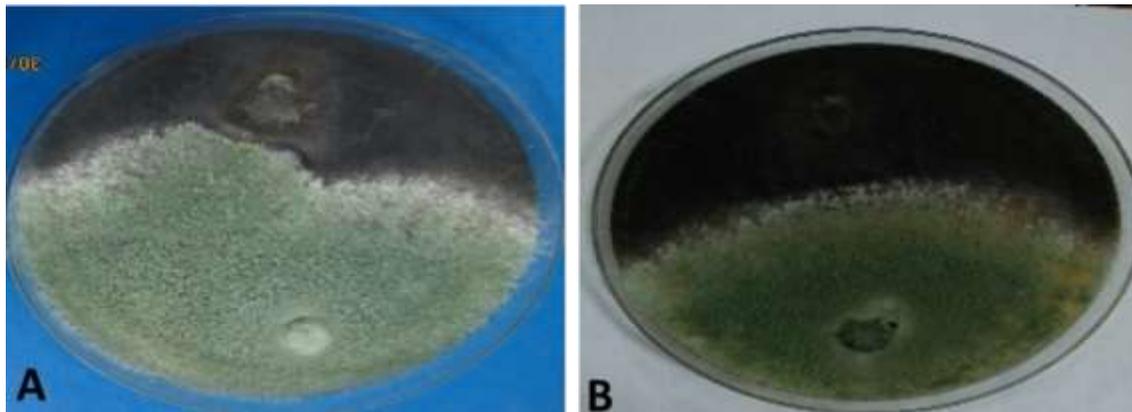
The antagonism of *Trichoderma* isolates against *M. phaseolina* and *A. niger* was evaluated *in vitro* by measuring the radial growth inhibition of the two pathogens (Table 2 and Figure 3).

The isolates Th2, Ta2, Th3 and TI were the most efficient isolates in suppression growth of *M. phaseolina*

**Table 2.** Radial growth inhibition of *Trichoderma* isolates against *M. phaseolina* and *A. niger* in dual culture plate technique

Isolates code	The radial growth inhibition of <i>M. phaseolina</i> (%)	The radial growth inhibition of <i>A. niger</i> (%)
Ta1	64.29 <sup>bc</sup>	57.14 <sup>d</sup>
Ta2	71.43 <sup>a</sup>	50.00 <sup>e</sup>
Ta3	50.00 <sup>d</sup>	42.85 <sup>f</sup>
Ta4	49.04 <sup>d</sup>	62.85 <sup>bc</sup>
Th1	54.29 <sup>c</sup>	54.28 <sup>de</sup>
Th2	72.85 <sup>a</sup>	64.28 <sup>b</sup>
Th3	67.14 <sup>b</sup>	58.57 <sup>cd</sup>
Th4	52.85 <sup>cd</sup>	57.14 <sup>d</sup>
Th5	25.71 <sup>e</sup>	71.43 <sup>a</sup>
Tl	65.69 <sup>b</sup>	31.43 <sup>g</sup>

Values followed by the same letter do not differ significantly ( $P=0.05$ ) according to Duncan's multiple range test". Results are means of 3 replicates for each treatment.

**Figure 3.** Antagonism of *T. harzianum* against *M. phaseolina* (A) and *A. niger* (B).

with 72.85, 71.43, 67.14 and 65.69%, respectively. In addition, the isolate Th5 gave only 25.71% radial growth inhibition against *M. phaseolina*. For antagonism of *A. niger*, the isolates Th5 and Th2 were the most active isolates in suppression growth of *A. niger* with 71.43 and 64.28% respectively, while the isolate Tl exhibited the least radial growth inhibition against the same pathogen (31.43%). In general, the isolate Th2 of *T. harzianum* showed good antagonism against *M. phaseolina* and *A. niger* with inhibition rates 72.85 and 64.28%, respectively. Previous studies explained that *T. harzianum* is more vigorous in inhibition growth of pathogenic fungi than the other species of *Trichoderma* (Abdel-lateif et al., 2017; Shoaib et al., 2018; Al-Hazmi and Javeed, 2016). *Mycoparasitism* is one of *Trichoderma* biocontrol mechanisms that depend on secretion of lytic enzymes like chitinases and cellulases to destroy the cell walls of pathogens (Gajera et al., 2012). It was proved that chitin is major cell wall component of most pathogenic fungi and *Trichoderma* is excellent producer of chitinases

which can degrade the chitin and therefore killing them (Benítez et al., 2004; Agrawal and Kotasthane, 2012; Gajera et al., 2012).

*Trichoderma* genus is proved to be an excellent control agent in inhibition growth of *M. phaseolina*, *Aspergillus* spp. and root knot nematode under *in vitro* and green house conditions (Al-Hazmi and Javeed, 2016; Khaledi and Taheri, 2016; Khalili et al., 2015; Mendoza et al., 2015; Shoaib et al., 2018; Athira, 2017; Krishnamurthy and Shashikala, 2006). The variability in aggressiveness of the same *Trichoderma* isolates against *M. phaseolina* and *A. niger* may be due to the variation in pathogen cell wall structure and the defense ability of the pathogen.

#### The effect of *Trichoderma* isolates on nematode

The efficacy of *Trichoderma* isolates against *M. incognita* on tomato was evaluated in greenhouse experiments and the results were compared with those of vydate and

**Table 3.** The effect of *Trichoderma* isolates on nematode growth parameters

<i>Trichoderma</i> isolate	No. of J2/250 g soil	Reduction (%)	No. of galls	Reduction (%)	No. of egg masses	Reduction (%)	No. of females	Reduction (%)
Ta1	21.33 <sup>e</sup>	90.33	16 <sup>gh</sup>	90.59	16 <sup>g</sup>	90.06	19 <sup>f</sup>	85.50
Ta2	25.66 <sup>de</sup>	88.37	20 <sup>efg</sup>	88.24	21 <sup>f</sup>	86.96	25 <sup>e</sup>	80.92
Ta3	29.00 <sup>cde</sup>	86.86	22 <sup>def</sup>	87.06	22 <sup>f</sup>	86.34	27 <sup>de</sup>	79.39
Ta4	35 <sup>bc</sup>	84.14	24 <sup>cde</sup>	85.88	30 <sup>de</sup>	81.37	33 <sup>c</sup>	74.81
Th1	24.00 <sup>de</sup>	89.12	19 <sup>fg</sup>	88.82	20 <sup>f</sup>	87.58	21 <sup>f</sup>	83.97
Th2	30.66 <sup>cd</sup>	86.11	22 <sup>def</sup>	87.06	24 <sup>f</sup>	85.09	28 <sup>de</sup>	78.63
Th3	35.33 <sup>bc</sup>	83.99	25 <sup>cd</sup>	85.29	32 <sup>cd</sup>	80.12	35 <sup>bc</sup>	73.28
Th4	39.66 <sup>b</sup>	82.03	27 <sup>c</sup>	84.12	34 <sup>bc</sup>	78.88	36 <sup>bc</sup>	72.52
Th5	40.33 <sup>b</sup>	81.72	35 <sup>b</sup>	79.41	37 <sup>b</sup>	77.02	37 <sup>b</sup>	71.76
TI	34b <sup>c</sup>	84.59	23 <sup>cdef</sup>	86.47	28 <sup>e</sup>	82.61	29 <sup>d</sup>	77.86
vydate	21.00 <sup>e</sup>	90.48	14 <sup>h</sup>	91.76	14 <sup>g</sup>	91.30	12 <sup>g</sup>	90.84
Nematode alone	220.66 <sup>a</sup>	0.00	170 <sup>a</sup>	0.00	161 <sup>a</sup>	0.00	131 <sup>a</sup>	0.00

Values followed by the same letter do not differ significantly ( $P=0.05$ ) according to Duncan's multiple range test<sup>ii</sup>. Results are means of 3 replicates for each treatment.

**Table 4.** The effect of *Trichoderma* isolates on tomato plant growth parameters.

<i>Trichoderma</i> isolate	Plant height (cm)	Root length (cm)	Fresh root weight (g)	Fresh shoot weight (g)	Dry shoot weight (g)
Ta1	80.76 <sup>b</sup>	29.90 <sup>b</sup>	7.64 <sup>b</sup>	76.44 <sup>a</sup>	8.99 <sup>b</sup>
Ta2	78.63 <sup>b</sup>	26.80 <sup>bc</sup>	6.77 <sup>bc</sup>	64.85 <sup>b</sup>	8.24 <sup>bcd</sup>
Ta3	78.00 <sup>b</sup>	24.46 <sup>cd</sup>	6.75 <sup>bc</sup>	55.55 <sup>c</sup>	7.80 <sup>cde</sup>
Ta4	70.86 <sup>c</sup>	17.96 <sup>fg</sup>	5.64 <sup>cd</sup>	47.89 <sup>cde</sup>	6.58 <sup>ef</sup>
Th1	79.13 <sup>b</sup>	27.16 <sup>bc</sup>	6.89 <sup>b</sup>	70.83 <sup>ab</sup>	8.8 <sup>bc</sup>
Th2	72.96 <sup>c</sup>	23.00 <sup>de</sup>	6.70 <sup>bc</sup>	53.42 <sup>c</sup>	7.34 <sup>def</sup>
Th3	70.43 <sup>c</sup>	16.63 <sup>gh</sup>	5.59 <sup>cd</sup>	43.53 <sup>de</sup>	5.47 <sup>g</sup>
Th4	64.96 <sup>d</sup>	14.86 <sup>gh</sup>	5.54 <sup>cd</sup>	40.46 <sup>ef</sup>	5.20 <sup>gh</sup>
Th5	60.33 <sup>d</sup>	14.50 <sup>gh</sup>	4.62 <sup>d</sup>	39.97 <sup>ef</sup>	5.12 <sup>gh</sup>
TI	72.30 <sup>c</sup>	20.73 <sup>ef</sup>	6.52 <sup>bc</sup>	50.27 <sup>cd</sup>	6.79 <sup>ef</sup>
vydate	88.33 <sup>a</sup>	34.63 <sup>a</sup>	9.49 <sup>a</sup>	77.02 <sup>a</sup>	10.48 <sup>a</sup>
Nematode alone	43.7 <sup>f</sup>	10.66 <sup>i</sup>	2.26 <sup>e</sup>	22.57 <sup>g</sup>	3.61 <sup>i</sup>
Control	52.03 <sup>e</sup>	14.03 <sup>h</sup>	3.24 <sup>e</sup>	33.39 <sup>f</sup>	4.34 <sup>hi</sup>

Values followed by the same letter do not differ significantly ( $P=0.05$ ) according to Duncan's multiple range test<sup>ii</sup>. Results are means of 3 replicates for each treatment

control. The results show that treatment of tomato seedlings with *Trichoderma* decreased significantly root galling, egg masses, number of females and second stage juveniles (J2) per root system (Table 3), while improved tomato growth parameters (root length, plant height, roots and shoots fresh weight and shoots dry weight) as indicated in Table 4. The isolates of *T. asperellum* (Ta1, Ta2 and Ta3) and the isolates of *T. harzianum* (Th1 and Th2) exhibited high inhibition of nematode growth in levels comparable with those of vydate. The isolate of *T. asperellum* (Ta1) was the best efficient isolate in reduction of each second stage juveniles (J2), number of galls, egg masses and females

per root system with 90.33, 90.59, 90.06 and 85.50%, respectively and the effect of this isolate was similar to vydate. Wu et al. (2017) isolated new strain of *T. asperellum* with high antagonism against pathogens of cucumber wilt and corn stalk rot.

On the contrast, the isolate *T. harzianum* (Th5) was the least isolate in reduction of each second stage juveniles (J2), number of galls, egg masses and females per root system with 81.72, 75.41, 77.02 and 71.76%, respectively, as compared to vydate. It was shown that the growth of nematode was increased in absence of *Trichoderma*. These results highlight the significance of *Trichoderma* as excellent control agent in inhibition growth

of nematode (Spiegel et al., 2007; Chen and Dickson, 2004; Sharon et al., 2011). Interestingly, most of the isolates that exhibited high inhibition rate of nematode growth also improved each root length, plant height, roots fresh weight, shoots fresh, and dry weight of tomato plants (Table 4). These results are compatible with other studies showing the ability of *Trichoderma* in growth inhibition of nematode on various crops and enhancing the growth of host plants (Al-Hazmi and Javeed, 2016; Izuogu and Abiri, 2015; Sharon et al., 2011; Mascarin et al., 2012).

The variability among *Trichoderma* isolates in their aggressiveness against the tomato nematode may be due to the difference in their genetic structure, origin of isolates, pathogen cell wall structure and quantity of lytic enzymes secreted by *Trichoderma* (Al-Hazmi and Javeed, 2016; Abdel-lateif et al., 2017).

## Conclusion

Identification of *Trichoderma* based on ITS markers exhibited high efficiency in discrimination among different *Trichoderma* spp. isolates. *Trichoderma* isolates showed variability in their aggressiveness against *M. phaseolina*, *A. niger* and *M. incognita*. In general, the isolate Th2 of *T. harzianum* showed the best antagonism against *M. phaseolina* and *A. niger*, while the isolate Ta1 of *T. asperellum* was the best efficient isolate in reduction of nematode growth.

In general, treatment of tomato with *Trichoderma* isolates improved their growth parameters (root length, plant height, roots and shoots fresh weight and shoots dry weight) as compared to untreated control. These results confirm the efficacy of *Trichoderma* as excellent biocontrol agent and also as plant growth promoting.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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*Full Length Research Paper*

# **Antimicrobial resistance pattern of *Acinetobacter* spp. isolated from clinical samples in a tertiary care hospital at Madinah, Saudi Arabia**

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**Multidrug resistance, in rapidly spreading bacterial species, is a matter of concern in health care units. Samples (6840) collected from a Saudi hospital in Madinah, were screened for *Acinetobacter* spp. and studied for frequency, gender distribution, and seasonal variations besides antimicrobial resistance pattern. *Acinetobacter* strains represented 5.5% of the clinical isolates from different sources. Of these, 63% were recovered from male samples. The largest numbers of these organisms were isolated from sputum and wound swabs representing about 85% of the samples. Antimicrobial sensitivity pattern showed that colistin was the most effective drug since it inhibited 76% of the *Acinetobacter* isolates. The remaining drugs did not reveal sensitivity greater than 15%. Results suggest colistin to be a promising drug in the treatment of *Acinetobacter* infections. It was also observed that percentage of these infections was highest during summers representing 39.15%, followed by autumn (28.17%) and winters (26.48%), while spring season recorded the least percentage with only 6.2% of infections. The present study was an attempt to generate data on the prevalence of resistance pattern and management of *Acinetobacter* infections.**

**Key words:** *Acinetobacter*, antimicrobial sensitivity pattern, multidrug resistance, nosocomial infections.

## **INTRODUCTION**

*Acinetobacter* species are gram negative bacteria that belong to the family Moraxellaceae. These encapsulated

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cocco-bacilli rods are non-motile, aerobic and do not ferment glucose. They test positive for catalase and citrate but negative for indole and oxidase (Manchanda et al., 2010; Almasaudi, 2018). They generally form part of the normal body flora but can cause a wide variety of local and systemic infections like pneumonia, septicemia and wound infections (Beggs et al., 2006; Almasaudi, 2018). The species can often be isolated from reusable medical equipments such as ventilator tubing, artificial pressure monitoring devices, humidifiers and wash basins. Gram-negative bacteria have the ability to acquire drug resistance genes such as extended-spectrum-lactamases (ESBLs), AmpC- $\beta$ -lactamase, and carbapenemases (Al-Arfaj et al., 2011; Bader et al., 2017).

*Acinetobacter* spp., especially *A. baumannii*, have been described as the leading nosocomial pathogen and is prevalent world-wide (Abbo et al., 2005; Kanafani and Kanj, 2014). It is responsible for high mortality rates in critically ill patients (Doughari et al., 2011). It is found more frequently in hospital ICUs, than *Staphylococcus aureus* and *Pseudomonas* spp. (Almasaudi, 2018). *A. baumannii* is considered the "red alert" pathogen by the Infectious Diseases Society of America as it has significantly threatened the efficacy of the present antibacterial (Peleg et al., 2008). Several cases of drug resistant *A. baumannii* have been reported from hospitals in UAE, Bahrain, Saudi Arabia, Palestine and Lebanon (Almasaudi, 2018). Studies have shown that the most frequently isolated organism from ICU patients of tertiary care hospitals in Riyadh, Saudi Arabia is *A. baumannii* (Saeed et al., 2010; Kamolvit et al., 2015). High infection rates have been reported from the southern region of Saudi Arabia (Al Bshabshe et al., 2016; Almaghribi et al., 2018).

At present, antibiotics are used frequently in diverse fields. They are prescribed to take care of unwanted infections that may occur during surgeries, organ transplants, and for the treatment of chronic infections (Teillant et al., 2015). Antibiotics are also used in animal husbandry and agriculture. To be precise, knowingly or unknowingly, there is exposure to different antibiotics which besides being toxic lead to multidrug resistance (MDR) and high treatment expenditures (Zowawi, 2016). MDR has already become a health issue at global level and it has been estimated that by the year 2050 it will be the leading cause of maximum deaths. MDR is common in Gram-negative bacteria and unfortunately there is scarcity of drugs that can provide effective treatment (Prestinaci et al., 2015). Contemporary studies from Middle East have shown that MDR in microbes is increasing extensively (Alawi and Darvesh, 2016). Carbapenem-resistant *A. baumannii* from Saudi Arabia have also increased dramatically over the years (Zowawi, 2016). A recent study showed that Saudi diabetic patients were significantly more likely to carry carbapenem-

resistant isolates of this species (Alsultan et al. 2013). It has now become crucial to deal with MDR and this is possible by first being aware of the perils involved and then formulating and complying with appropriate guidelines (Baadani et al., 2015; Al-Harathi et al., 2015). The present study is an effort to determine the prevalence and antimicrobial susceptibility pattern of *Acinetobacter* spp. from clinical sources at a busy hospital in Madinah, Saudi Arabia.

## MATERIALS AND METHODS

### Sample collection

Different clinical samples such as sputum, wound swab, bile, tracheal aspirate (Tr. asp.), throat aspirate (Th. asp.), catheter tip, pus, abdominal abscess (Abd. ab.), ear swab, peritoneal wound swab (Peri. w.s.), pleural fluid (Pler. fluid), vaginal swab (VS), urethral discharge (UD), eye cornea swab (ECS), bone tissues, and brain tube were collected from 6840 patients suspected of nosocomial bacterial infection at King Fahd Hospital at Madinah, Saudi Arabia according to Centers for Disease Control and Prevention Specimen Collection Guidelines (CDCP, 2013). Demographic data such as sex of the patients was recorded prior to sample collection.

### Culture and Identification

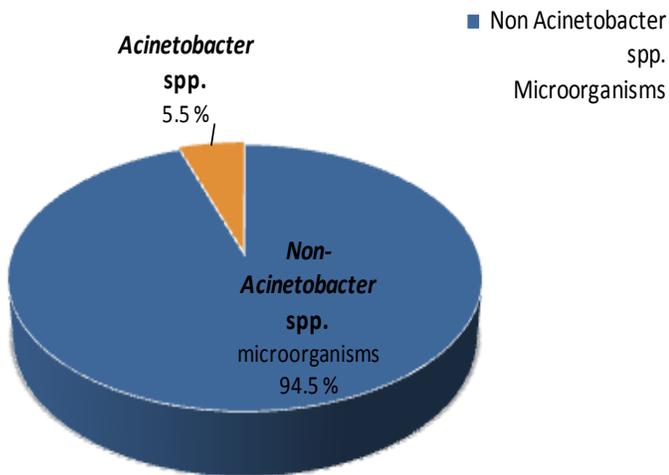
The clinical samples were collected, aseptically inoculated on plates of blood agar, chocolate agar, cystine-lactose-electrolyte-deficient (CLED) agar and MacConkey agar (Oxoid Cambridge, UK) and incubated at 37°C for 24 h. Identification was done based on morphological characteristics of the colonies including size, shape, colour, pigmentation and haemolytic nature (Cheesbrough, 2006).

### Biochemical characteristics

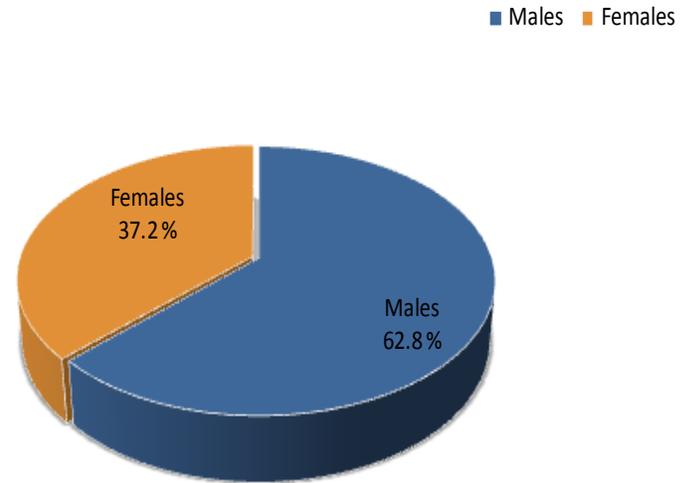
Suspected colonies of *Acinetobacter* were further identified through biochemical tests (Barrow and Feltham, 2003) using standard procedures and Phoenix automated microbiology 100 ID/AST system (Becton Dickinson Company, Sparks, Md.).

### Antimicrobial susceptibility test

Susceptibility to antimicrobial agents was determined by using the disk diffusion method (Oqunshie, 2006), and Phoenix automated microbiology 100 ID/AST system (Becton Dickinson Company, Sparks, Md.). The following antimicrobial agents (obtained from BDH London, UK) were used: augmentin [amoxicillin + clavulanic acid (20/10  $\mu$ g)], gentamycin (10  $\mu$ g), cefoxitin (30  $\mu$ g), cephalothin (30  $\mu$ g), cotrimoxazole [trimethoprim-sulfamethoxazole 1:19 (25  $\mu$ g)], amikacin (30  $\mu$ g), ceftazidime (30  $\mu$ g), aztreonam (30  $\mu$ g), piperacillin (100  $\mu$ g), imipenem (10  $\mu$ g), ciprofloxacin (5  $\mu$ g), cefpiramide (30  $\mu$ g), tazobactam (10  $\mu$ g), colistin (10  $\mu$ g), and tigecycline (15  $\mu$ g). The inocula were prepared by growing the strains on separate agar plates and colonies from the plates were transferred with a loop into 3 ml of normal saline. The density of these suspensions was adjusted to 0.5 McFarland standard. The surface of Muller-Hinton agar (Oxoid Cambridge, UK) plate was evenly inoculated with the organisms using sterile swabs. The swab was dipped into the suspension and pressed against the side of the



**Figure 1.** Percentage of *Acinetobacter* spp. in comparison to other clinical isolates.



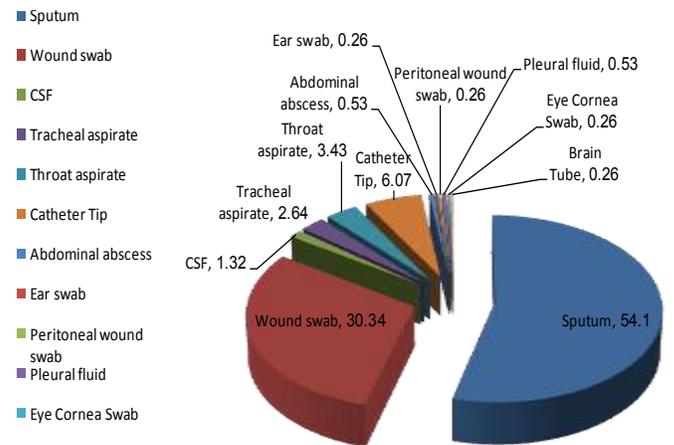
**Figure 2.** Percentage isolates of *Acinetobacter* spp. found positive in males and females.

test tube to remove excess fluid. The wet swab was then used to inoculate the Muller-Hinton agar by evenly streaking across the surface. By means of a Disc Dispenser (Oxoid Cambridge, UK), the antibiotic discs were placed on the surface of the inoculated agar and the plates were incubated overnight at 37°C. The diameter of zone of growth inhibition observed was measured and compared to the chart provided by Clinical and Laboratory Standards Institute (CLSI, 2017).

**RESULTS AND DISCUSSION**

Due to ever increasing drug resistance, not many effective antimicrobials are available for the treatment of nosocomial *Acinetobacter* infections. Hence, there is an ardent need to develop new drugs and re-evaluate older antimicrobial therapies (Jain and Danziger, 2004). The risk factors that lead to infection with resistant *Acinetobacter* spp. are: prolonged stay in hospital (especially ICUs), mechanical ventilation, exposure to antimicrobial agents especially carbapenems, recent surgeries, invasive procedures and chronic illnesses (Manchanda et al., 2010). Self-medication and careless prescriptions given by doctors make matters worse. Several studies have reported the antimicrobial resistance patterns in Saudi Arabia, but there are only a few reports from Mecca and Madinah (Rotimi et al., 1998; Al-Johani et al., 2010; Zowawi, 2016). These two cities are visited by a large population of pilgrims every year. The present study is an endeavor to understand the resistance pattern of *Acinetobacter* spp. to several antimicrobial drugs. Frequency, gender distribution and seasonal variations were also studied with respect to infection caused by these bacteria.

Figure 1 shows the percentage of *Acinetobacter* spp. in comparison to other clinical isolates. These bacterial



**Figure 3.** Percentage of *Acinetobacter* spp. isolated from different clinical samples.

species comprised 5.5% (379 strains) of the total isolates; 62.8% (238 strains) were from male patients and 37.2% (141 strains) were from female patients (Figure 2). A higher number of isolates from male patients indicate greater prevalence of these infections in males than females. This pattern has also been displayed in several previous studies (Abbo et al., 2005; Masoud et al., 2011; Magliano et al., 2012; Howard et al., 2012; Haseeb et al., 2016). Figure 3 shows the percentage of *Acinetobacter* spp. recovered from various clinical sources. Pus, bile, ascitic fluid, vaginal swabs, urethral discharge, semen, bone tissue, appendix, gall bladder aspirates, cystic fluid, necrotic tissue were some samples from which the

**Table 1.** Gender-wise estimation of the number of *Acinetobacter* spp. isolated from different sources.

Sex	Specimen sources											
	Sp	WS	CSF	Tr	Th	Cath	Abd	Ear	Peri	Pler	ECS	Brain Tube
Male	N:121(59%)	N:74 (64.3%)	N:4 (80%)	N:8 (80%)	N:7 (53.8%)	N:17 (74%)	N:2 (100%)	N:1 (100%)	N:1 (100%)	N:1 (50%)	N:1 (100%)	N:1 (100%)
Female	N:84 (41%)	N:41 (35.7%)	N:1 (20%)	N:2 (20%)	N:6 (46.2%)	N:6 (26%)	N:0 (0%)	N:0 (0%)	N:0(0%)	N:1 (50%)	N:0 (0%)	N:0 (0%)
Total (N)	205	115	5	10	13	23	2	1	1	2	1	1

Sp, Sputum; WS, wound swab; CSF, cerebrospinal fluid; Tr, tracheal aspirate; Th, throat aspirate; Cath, catheter Tip; Abd, abdominal abscess; Peri, peritoneal wound swab; Pler, pleural fluid; ECS, eye cornea swab.

*Acinetobacter* spp. were not recovered. Majority of the isolates were from sputum (54.1%) and wound swabs (30.34%). Catheter tips, throat aspirates, tracheal aspirates and cerebrospinal fluids constituted 6.07, 3.43, 2.64 and 1.32%, respectively. Abdominal abscess and pleural fluid contributed only 0.53% each while ear swabs, peritoneal wound swabs and brain tube each provided the lowest percentage of 0.26%.

Table 1 provides an estimation of the gender distribution of samples isolated from different sources. As mentioned above, the major sample sources of the *Acinetobacter* spp. were sputum and wound swabs comprising 84.44% of the isolates. Out of 205 sputum samples, 59% were from males and 41% were from females. In the case of wound swabs (115 samples), 64.3% were from males and 35.7% were from females. Similarly, in case of cerebrospinal fluid and tracheal aspirates, although the samples were less in number, the male to female percentage ratio was 80:20. In case of catheter tips, again 74% samples were from males and 26% were from females. Only 13 samples were available from throat aspirates and were in the ratio of 53.8 males to 46.2 females. Pleural fluid provided only 2 strains, one each from male and female. Ear,

peritoneal wound swab, eye cornea swab and brain tubes provided only 1 sample each and that also from males. As the sample number from these sources was low, significant conclusions could not be drawn from them. The results hence indicated that males are more prone to infection than females. Lifestyle differences of both the genders and social and economic factors have an influence on the infection pattern. In Saudi Arabia, since women live a protected life usually indoors, their chance of acquiring bacterial infections is usually low in comparison to males. This pattern has been reported by other researchers as well (Haseeb et al., 2016; Magliano et al., 2012).

Antimicrobial drug susceptibility pattern of *Acinetobacter* spp. was assessed for 15 antimicrobial drugs listed in Table 2. All the drugs showed low sensitivity for *Acinetobacter*. Highest sensitivity was shown by colistin (76%), followed by amikacin (14.78%), gentamycin (12.9%) and imipenem (10.82%). For the remaining antimicrobials, the percentage was below 7% being significantly low. Highest resistance was shown towards cephalothin (99.74%), tazobactam (99.48%), augmentin (99.21%) and cefoxitin (99.21%). The percentage resistance of *Acinetobacter* spp. for other antimicrobials was in

the following order: tigecycline (98.96%) > ceftazidime (98.42%) > cefpiramide (98.42%) > ciprofloxacin (98.15%) > aztreonam (97.89%) > piperacillin (97.63%) > cotrimazole (93.14%) > imipenem (89.18%) > gentamycin (87.1%) > amikacin (85.22%). Colistin showed comparatively low resistance of only 24%. The results of the present study are coordination with the previous findings (Fishbain and Peleg, 2010). A study conducted in the southern region of Saudi Arabia showed that 74% of *Acinetobacter* isolates had MDR of which 50% were sensitive to colistin. These were resistant to all the other antimicrobials studied (Falagas et al., 2015; Almaghrabi et al., 2018).

Bacterial infections occur throughout the year but show seasonal variations depending upon several factors (Fares, 2013). These factors have to be explored to understand the prevalence and prognosis of the infections and to evolve novel and improved infection control strategies. There are several reports claiming that bacterial infections always peak during summers and winters (Perencevich et al., 2008; Eber et al., 2011; Richet, 2012). Table 3 shows the infection pattern of *Acinetobacter* during four different seasons of the year in the city of Madinah.

**Table 2.** Percentage (%) of antimicrobial sensitivity pattern of *Acinetobacter* spp. to different antibiotics.

Antibiotic	Sensitive	Resistant	Intermediate
Augmentin	0.79	99.21	0
Gentamycin	12.9	87.1	0
Cefoxitin	0.79	99.21	0
Cephalothin	0.26	99.74	0
Cotrimoxazole	6.86	93.14	0
Amikacin	14.78	85.22	0
Ceftazidime	1.58	98.42	0
Aztreonam	2.11	97.89	0
Piperacillin	2.37	97.63	0
Imipenem	10.82	89.18	0
Ciprofloxacin	1.85	98.15	0
Cefpiramide	1.58	98.42	0
Tazobactam	0.52	99.48	0
Colistin	76.0	24.00	0
Tigecycline	1.04	98.96	0

**Table 3.** Percentage (%) of *Acinetobacter* infection pattern during different seasons.

Season	Percentage (%) of infections
Summer (22 <sup>nd</sup> June -22 <sup>nd</sup> September)	39.15
Autumn (23 <sup>rd</sup> September -21 <sup>st</sup> December): Pilgrimage	28.17
Winter (22 <sup>nd</sup> December -30 <sup>th</sup> March)	26.48
Spring (21 <sup>st</sup> March -21 <sup>st</sup> June)	6.20

Infections occurred with highest frequency in summers at 39.15 %. In winter season the infection was comparatively low (26.48 %). This maybe because the autumn season, that follows winters, coincides with the annual Hajj pilgrimage. This season experiences a huge influx of people into the two holy cities of Makkah and Madinah. During this event special care is taken by the health authorities to monitor and control any outbreak. As a result, low percentage of infections is recorded. A pattern of high infection rates in summers followed by slightly lower rates in the pilgrimage season have also been observed in the case of other Gram negative bacteria namely *Proteus* (Bahashwan and Shafey, 2013), *Klebsiella* (Ghanem et al., 2017), *Pseudomonas* (Saeed et al., 2018a) and *E. coli* (Saeed et al., 2018b) during the same period of study.

The two holy cities of Makkah and Madinah are visited by people from all around the world for Umrah and Hajj. Although Hajj is performed in a particular season, Umrah is performed all year round. It is a huge challenge for health authorities to keep infections under control. The enormous amount of people visiting at the same time from different places can lead to uncontrollable epidemics.

Special precautions taken by the health care authorities help to keep infections and antimicrobial drug resistance in control. Several infection control programs are being implemented like the World Health Organization (WHO) hand hygiene program, the Gulf Cooperation Council (GCC) Infection Control Program (Yezli et al., 2014) and the antimicrobial stewardship program (Alawi and Darwesh, 2016; Zowawi, 2016). These programs are well coordinated into providing safe and cost-effective strategies for improved patient outcomes and reduced antimicrobial resistance.

## Conclusion

Sputum samples followed by wound swabs were found to be the largest source of *Acinetobacter* isolates. Samples isolated from male patients were a greater source in comparison to the female patients, maybe because males are at a greater risk to infection, being the main work force in Saudi Arabia. None of the tested antimicrobials showed 100% sensitivity. The highest sensitivity was shown by colistin at 76% followed by

amikacin, gentamycin and imipenem. The rest of the antimicrobials showed a sensitivity of less than 7%. Highest resistance was displayed by cephalothin, tazobactam, augmentin and cefoxitin in the same order. These antimicrobials should be prescribed with care. The infections occurred with highest frequency in summers and lowest in spring. The frequency dropped in autumn as it coincided with the Hajj season when authorities are extra cautious. The present study will help in generating local database that can be used for formulating infection control strategies in this area, especially during pilgrimage season. People should get appropriate education on the issue of MDR, especially physicians who should develop a sense of responsibility while prescribing antibiotics. Similar studies will contribute towards creation of effective guidelines and infection control programs.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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*Full Length Research Paper*

# Prevalence and genetic diversity of the strains of *Bacillus cereus* groups in food for infants and young children in México

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The aim of this study was to determine the prevalence and genetic diversity of the strains of *Bacillus cereus* groups isolated in México from foods for infants and young children. A total of 94 foods from a single commercial brand were analyzed to find *B. cereus* through a pre-enrichment method of colonial morphology in agar mannitol yolk polymyxin. Specific colonies were selected to be analyzed by polymerase chain reaction (PCR) determining the amplification of the 16S *rRNA* gene from *B. cereus* and the cytotoxin K gene. Eight strains were selected to determine genetic diversity (and relation) between the isolates from a PCR of repeated elements (rep-PCR), technique of molecular phylogeny that uses the primer (GTG) 5. The genetic similarity was determined by the Dice coefficient and from this a dendrogram was carried out. Isolates corresponding to strains from *B. cereus* group were detected in 9.2% (8/92) of the samples analyzed, 87.5% of the eight isolates showed the *cytK* gene. Groups of isolated strains were detected in meats and fruits and there was only one isolated strain from vegetables. The study shows the presence and propagation of strains from *B. cereus* group in foods for infants and young children commercialized in México.

**Key words:** *Bacillus cereus*, México, genetic diversity, enterotoxin, infant food.

## INTRODUCTION

Foods for infants and children are mainly used during the normal weaning period and during the gradual adaptation of infants and children to normal feeding. They are prepared to be directly administered or dehydrated to be reconstructed through water dilution. The microbiological criteria for these foods state that they must be free from

pathogenic microorganisms or self-produced substances in amounts that could cause disease (CAC, 1979; FAO/WHO, 1981).

The *Bacillus cereus* group is made up of six species of bacteria closely related: *B. cereus sensu stricto*, *Bacillus anthracis*, *Bacillus mycoides*, *Bacillus pseudomycoides*,

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*Bacillus thuringiensis* and *Bacillus weihentephanensis* (Vilas-Bôas et al., 2007). This group of bacteria is spore-forming Gram-positive bacilli with a wide spread in the environment, decomposing organic matter, fresh or salty water bodies, fomites, and naturally in the gastrointestinal tract of invertebrate organisms. Foods may be contaminated by these reservoirs and colonize the human intestine upon ingestion (Samapundo et al., 2011).

Some species from the *B. cereus* group may cause food poisoning due to the production of different toxins: cereulide is a thermostable toxin resistant to acid pH and proteases, composed of four amino acids and/or oxyacids [D-O-Leu-D-Ala-L-O-Val-L-Val] that form a cyclic complex (dodecadepsipeptide) (Stenfors-Arnesen et al., 2008). This toxin causes the afferent vagus nerve stimulation through the attachment to 5-HT<sub>3</sub> receptor of serotonin, causing the emetic syndrome characterized by nausea and vomit. The main related foods are rice, pasta and dairy products. The presence of the preformed toxin in the foods is enough to cause the disease (Delbrassine et al., 2012; Dommel et al., 2010).

In contrast, diarrhea toxins like hemolysin BL (Hbl), non-hemolytic enterotoxin (Nhe) and cytotoxin K (CytK) are produced during the vegetative growth of the bacteria in the small intestine (Naranjo et al., 2011). The Hbl and Nhe enterotoxins are made up of three subunits; L2, L1B, and NheA, NheB, and NheC, respectively, whereas the CytK is made up of a single protein from the family of barrels  $\beta$  (Bottone, 2010). The three toxins have lytic activity against enterocytes, the mechanism is not precisely known, however, it suggests the formation of pores in the lipid membranes of cells, which leads to osmotic lysis (Stenfors-Arnesen et al., 2008; Tsilia et al., 2012).

A high frequency of isolated toxigenic strains from group *B. cereus* has been recently reported in different food groups, including foods rich in starch (rise and potatoes), raw or partially cooked vegetables, dairy and meat products, and ready-to-eat foods (Chon et al., 2012; Lee et al., 2012; Samapundo et al., 2011).

Diseases related to foods contaminated by species from group *B. cereus* generally occur when toxigenic strains multiply and reach around  $10^4$  to  $10^6$  CFU, however, due to the high variability of infectious doses, as well as toxin production and spore formation, it is not possible to rule out the risk of smaller size inoculates (Logan, 2012).

In 2015, in the United States of America, two food poisoning cases caused by *B. cereus* were reported with 25 people without going to hospital (Centers for Disease Control and Prevention (CDC), 2017). Meanwhile, in countries like Norway, Finland, and Hungary, *B. cereus* has been held accountable for gastrointestinal diseases (diarrhea syndrome) in contrast with countries like China, Japan, and Belgium, where it is linked to the emetic syndrome (Granum and Lund, 1997; Logan, 2012). The existence of *B. cereus* as a contaminant of food for

babies has been reported in China (Li et al., 2014; Zhang et al., 2017) and Iran (Rahimi et al., 2013), and currently, in Mexico there are no data about this microorganism. In Mexico, until March 2018, over 600 food poisoning cases and more than 90,000 gastrointestinal diseases have been reported without identifying the causal agent (Dirección General de Epidemiología, 2018). The aim of this study was to analyze and compare the *B. cereus* frequency in a wide array of foods for infants and small children, the presence of cytotoxin K gene, and possible strain cloning.

## MATERIALS AND METHODS

### Sampling and microbiological analysis

The products were chosen per the general description of foods for infants made by the Codex Alimentarius described earlier, considering in this study a total of 94 foods of a single commercial brand, which were purchased in supermarkets in Chilpancingo, a southwestern city from Mexico. Remarking that even when they were purchased in this city, they are widely distributed and purchased throughout the country. The samples were collected from January to July, 2017. The 94 samples comprised varieties fruit (35 samples), vegetables (32 samples) and meat (27 samples).

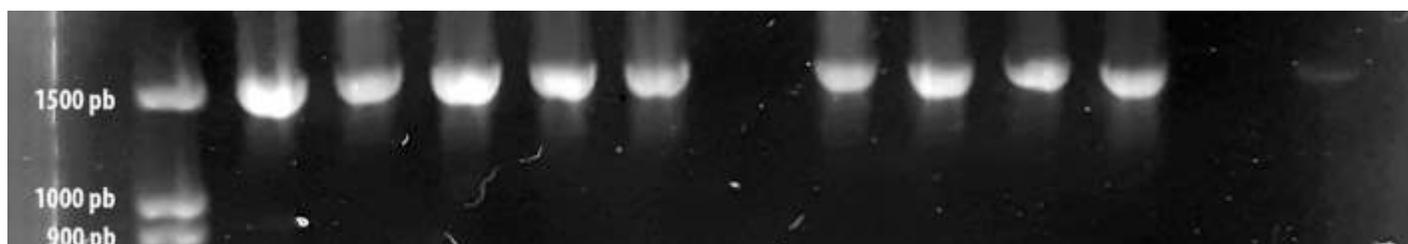
The microbiological analysis was limited to the count of mesophyll microorganisms in plaque and a pre-enrichment method for the research of *B. cereus*. For the mesophilic count, 25 g of the sample were used, which were taken aseptically and mixed in 225 mL of peptone saline solution. After 10 min, aliquots of 1 mL were taken and from these, decimal dilutions were made in sterile saline. The dilutions were inoculated by the dispersion method placing a volume of 0.1 mL on nutritive agar plates. The plates were incubated for 24 h at 30°C. The colonies were counted to determine the total count of aerobic microorganisms in plaque. From the remaining mixture in peptone saline solution, a 1 mL aliquot was transferred to a brain heart infusion broth supplemented with 10 U/mL of polymyxin and after incubation at 30°C, aliquots of 0.1 mL were inoculated in mannitol agar polymyxin egg yolk (MYP). The pink colonies with an opaque halo were considered as suspicious colonies of *B. cereus* and were confirmed by beta hemolysis in trypticase soy agar supplemented with sheep blood.

### Molecular confirmation of strains of the *B. cereus* group and presence of cytotoxin K

From bacterial cultures of the bacterial strains, a thermal shock was performed to obtain the chromosomal DNA. In brief, cells from one colony were suspended in sterile water, heated at 95°C for 3 min and then placed on ice. After centrifugation, the supernatant was used as template for the amplification of the 16S *rRNA* gene of *Bacillus*. The primer for the 16S *rRNA* gene amplification was derived from the data record GenBank: AE016877 for the reference strain *B. cereus* ATCC 14579. In order to amplify the 16S *rRNA* gene, the reaction mixes (25  $\mu$ L) contained the following: 25  $\mu$ L of REDTaq Ready Mix DNA polymerase (Sigma- Aldrich, USA), 11  $\mu$ L of sterile MiliQ water, 0.5  $\mu$ L of the genomic DNA template (concentration about 10 to 20 ng/ $\mu$ L) and 1  $\mu$ L of each primer (FRNA, 5-AGA GTT TGA TCC TGG CTC-3; RRNA, 5-CGG CTA CCT TGT TAC GAC-3). The reactions were carried out with an initial denaturation at 94°C for 1 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 3 min and the final extension at 72°C for 3 min. For the detection of the *cytK* gene, polymerase chain reaction (PCR) reaction was set up. The reaction mixes

**Table 1.** Aerobic plaque count and presence of *B. cereus* in different varieties of food for infants.

APC count (Log CFU/g)	Variety		
	Fruits [n (%) N=35]	Vegetable [n (%) N=32]	Meat [n (%) N=27]
1	24 (68.6)	23 (71.8)	9 (33.3)
2	6 (17.1)	9 (28.2)	11 (40.7)
3	3 (8.6)	0	4 (14.8)
4	2 (5.7)	0	3 (11.2)
<i>B. cereus</i>	2 (5.7)	1 (3.1)	5 (18.5)

**Figure 1.** Agarose gel electrophoresis of the PCR products obtained for selected strains from the *B. cereus* group with primers 16S *rRNA* gene. Lane 1, 100 bp molecular marker; Lane 2, *B. cereus* ATCC 14579, Lane 3-13, samples.

contained: 12.5  $\mu$ L of RedTaq Ready Mix DNA polymerase (Sigma-Aldrich, USA), 11.5  $\mu$ L of sterile MilliQ water, 0.2  $\mu$ L of each primer solutions (concentration 100  $\mu$ M) P1-5'-CAA AAC TCA (T/C) CTA TGC AAT TAT GCA T-3', P3-5'-ACC AGT TGT ATT AAT AAC GGC AATC-3'), 1  $\mu$ L of the template DNA with concentrations equaling 10 to 20 ng/ $\mu$ L. The amplification cycle composed of initial denaturation at 94°C for 2 min, followed by 40 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 1 min and final extension at 72°C for 2 min. Electrophoresis was performed on 2% agarose gels at 80 V for 120 min. The gels were stained with Midori Green (Nippon Genetics, Germany) and visualized with UV light.

#### Genetic diversity of *B. cereus*

The phylogenetic relationship between the strains of *B. cereus* was carried out using the technique of repetitive palindromic elements PCR (rep-PCR)<sup>13</sup> using primers (GTG) 5 (GTG GTG GTG GTG GTG) with the following reaction conditions; initial denaturation at 95°C for 2 min, 30 cycles of 94°C for 30 s, 40°C for 1 min, 60°C for 1 min and for a final extension, 65°C for 5 min. Electrophoresis was performed on 2% agarose gels at 80 V for 120 min. The gels were stained with Midori Green (Nippon Genetics, Germany) and visualized with UV light.

To establish the genetic distances of the profiles, the coefficient of similarity of Dice was calculated. The genetic distance matrix was analyzed by the UPGMA method (acronym in English of Unweighted Pair Group Method with Arithmetic Mean). A dendrogram was developed with the analyzed data using the BioNumerics 7.1 package (Applied Maths Inc, US).

#### Statistical analysis

Descriptive statistics are used to describe the basic features of the data in a study. They provide simple summaries about the sample and the measures.

## RESULTS

In the present study, numbers above 4.0 log CFU/g of aerobic mesophilic in foods was observed for infants of meat and fruit varieties. However, one of the most important results was the presence of *B. cereus* in all varieties of foods for toddlers (Table 1).

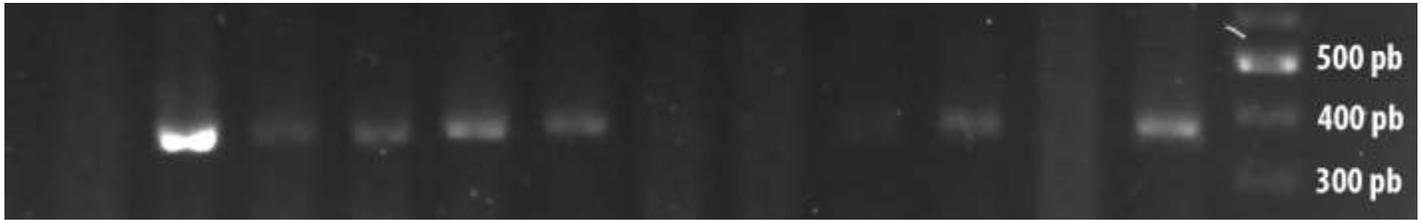
To confirm the proper classification of isolates, the amplification by PCR of the 16S *rRNA* gene was used. PCR amplification products of the nearly complete 16S *rRNA* gene were observed (1500 bp about) in eight strains (Figure 1). The results obtained for their strains confirmed their taxonomic position as belonging to the *B. cereus* group.

PCR was used for the detection of the *cytK* gene in eight strains belonging to the *B. cereus* group. An amplicon, 238 bp long was obtained containing the N-terminal fragment of the structural gene. It was found that among eight isolates, most of them (7 strains, 87.5%) contained the *cytK* gene (Figure 2)

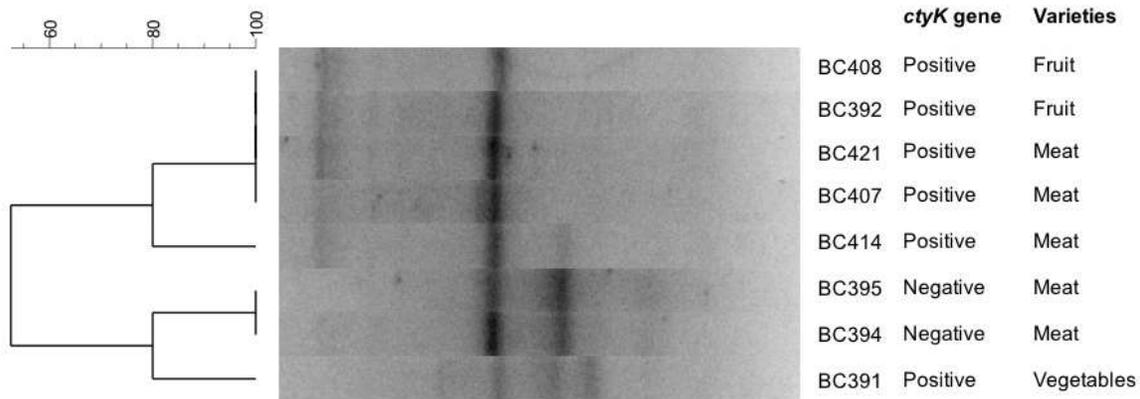
Upon identifying possible cloning of strains, four different groups could be observed; three of them positive for cytotoxin K. In one of them, an isolated vegetable strain was grouped, whereas isolated meat strains were distributed among the remaining groups. In the first group both meat and fruit strains were grouped (Figure 3).

## DISCUSSION

In recent years, the role and importance of *B. cereus* as a



**Figure 2.** Agarose gel electrophoresis of the PCR products obtained for selected strains from the *B. cereus* group with primers *cytK* gene. Lane 1, Negative control; Lane 2- 12, samples; Lane 13, *B. cereus* ATCC 14579; Lane 14, molecular marker.



**Figure 3.** Dendrogram (obtained by GTG5) of cloning of strains of the *B. cereus* group. Eight strains of processed foods for infants positive to 16s *rRNA* were characterized and grouped into four main groups.

pathogen associated with food poisoning has increased (Jeßberger et al., 2015). Even though risk groups have not been described, it has been reported that the infection is not self-limited in all cases and that it can even progress to death (Dierick et al., 2005; Lund et al., 2000).

In children under two years old, in addition to breastfeeding, different sorts of packaged foods are included in the diet, importantly; this group of foods is recognized as non-sterile, establishing internationally the microbiological parameters that these must meet (FAO/WHO, 1981).

At a national level, the Mexican regulations framework considers as a quality parameter the amount of CFU/g of aerobic mesophilic (Secretaria de Salud (SSA), 1995). In this study, five fruit samples and seven meat samples exceed this amount; an important feature in this regulation, is not included the amount of CFU/g or the presence of *B. cereus* as an important microbiological indicator, even when the population to which these products are directed, could be a risk group. In this study, *B. cereus* was isolated in all varieties with a frequency of 9.2%; in studies carried out in China, the prevalence ranges from 1 to 6% (Li et al., 2014; Zhang et al., 2017), while in Iran it is 42% (Rahimi et al., 2013), considering that the main difference is the type of variety analyzed, in

these three studies the base was rice and in the present study, it was rice-based foods, fruits and vegetables were included.

These data as a whole denote the capacity of the spores of the *Bacillus* genus to resist thermal treatments (wet and dry heat), radiation (UV and ionizing) and chemical agents (Setlow, 2006), treatments to which these products are subjected to their preparation and packaging, particularly sterilization (variety of meats) and airtight seal (varieties of vegetables and fruits) (SSA, 1995); even when only the presence was determined (not the quantity), it is an important fact because these foods are stored for long periods of time either in shops or at home, which can favor the growth of the microorganism to an infectious dose (Valero et al., 2003), which has been described in vegetable purees, where the aerobic spore increased  $7.8 \pm 0.1$  log CFU/g in the puree after five days at 20 to 25°C and the vegetative cells of *B. cereus* approximately  $6.4 \pm 0.5$  log CFU/g in the same period of time and temperature (Guinebretiere et al., 2003). In addition, minor inoculants should therefore not be ruled out due to the toxigenic potential of each strain (Logan, 2012).

*B. cereus* is a microorganism with a wide variety of virulence factors, including emetic toxin and the enterotoxins Hbl, NHE and CytK (Bottone, 2010). In this

study, the *cytK* gene was molecularly identified, because the presence of this gene has been determined in strains that caused food poisoning (and that are negative for NHE and Hbl) (Lund et al., 2000). The frequency in this study was 87.5%, which is like that reported in a study in Belgium in ready-to-eat foods (Samapundo et al., 2011).

The intention to identify a possible clone in this type of food was focused on the search for a clone that will be isolated from all varieties of these foods and that may be related to a systematic contamination during its production and processing, due to its persistence in the area and resistance of the spores produced. For example, certain characteristics of spores, such as hydrophobicity or the presence of exosporium favor their ability to adhere to surfaces involved in the food processing (Tauveron et al., 2006). Additionally, it has been observed that this microorganism is capable of producing biofilm, which protects both the spores and vegetative cells from disinfectants (Ryu and Beuchat, 2005); this could be explained with the strains that belong to the first generated group. However, the presence of strains, such as the strain isolated from vegetables, could explain that the contamination of this microorganism may not only be found in the process, but also in the raw material. Related to this, the presence of *B. cereus* in dairy products was shown from early stages of product processing, which include contamination of raw material (Svensson et al., 2004) or even in raw vegetables for the preparation of puree (Choma et al., 2000), which is capable of enduring thermal and pressure processes to which the food is subjected (Guinebretiere et al., 2003) and this could explain the presence of the strain found in vegetables and not in other food groups.

This study proposes the incorporation of this microorganism in the current Mexican legislation, due to the characteristics that allow it to be found in food, as well as virulence factors that could cause major food poisonings in the group to which these foods are directed.

## Conclusion

The study shows the presence and propagation of strains from *B. cereus* group in foods for infants and young children commercialized in México

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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