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Full Length Research Paper

Bioaccumulation of cadmium and lead by *Shewanella oneidensis* isolated from soil in Basra governorate, Iraq

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In the present study heavy metals resistant bacteria were isolated from soil collected from Al-Zubair district in Basra governorate south of Iraq. On the basis of morphological, biochemical, 16S rRNA gene sequencing and phylogeny analysis, the isolates were authentically identified as *Shewanella oneidensis* in addition to *Bacillus thuringiensis* and *Deinococcus radiodurans*. The minimal inhibitory concentration (MIC) of isolates against cadmium (Cd) and lead (Pb) was determined on solid medium. *S. oneidensis* showed significant resistance to high concentrations of Cd (1000 mgl^{-1}) and Pb (700 mgl^{-1}). The bioaccumilation capabilities of *S. oneidensis* for Cd and Pb were monitored at different ion concentrations and contact times. The transmission electron microscope (TEM) study confirmed the accumulation of Cd and Pb by *S. oneindensis* causing morphological changes.

Key words: *Shewanella oneidensis*, bioaccumulation, minimal inhibitory concentration, heavy metals, transmission electron microscope.

INTRODUCTION

Heavy metals play an important role in the metabolic processes of the biota, some of them are essential for organisms as micronutrients (cobalt, chromium, nickel, iron, manganese and zinc). They are involved in redox processes, to stabilize molecules through electrostatic interactions, as catalysts in enzymatic reactions, and regulating the osmotic balance. On the other hand, cadmium, mercury, lead, have no biological role and are harmful to the organisms even at very low concentration. However, at high levels, both of the essential and non-essential metals become toxic to the organisms (Rathnayake et al., 2010).

Cadmium is widespread and one of the most toxic soil contaminants released by mining and smelting activities, atmospheric deposition from metallurgical industries, incineration of plastics and batteries, land application of sewage sludge, and burning of fossil fuels (Tang et al., 2006). Cadmium is poisonous to plants, animals, and humans (Gupta and Gupta, 1998) and is listed as one of the 126 priority contaminants by the USEPA and as a human carcinogen by the International Agency for Research on Cancer (IARC, 1994). Thus cadmium

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pollution is presently attracting more attention from environmentalists worldwide.

Lead (II) is a heavy metal poison which forms complexes with oxo-groups in enzymes to affect nearly all steps in the process of hemoglobin synthesis and porphyrin metabolism. Toxic levels of Pb (II) in man have been associated with encephalopathy appropriations and mental delay (Ademorati, 1996). Conventional physico-chemical methods such as electrochemical treatment, ion exchange, precipitation, reverse osmosis evaporation and sorption (Kadirvelu et al., 2001, 2002) have been used for removing heavy metals but are economically expensive and have disadvantages. Bioremediation is a natural process which depends on bacteria, fungi and plants to altering pollutants as these organisms perform their normal life functions. These organisms have the ability of exploiting chemical contaminants as an energy source in their metabolic processes. Therefore, bioremediation affords alternative tool to destroy or reduce the risky contaminants through biological activity with an effective cost (Salem et al., 2012).

Microbial populations in metal polluted environments become metals resistant (Prasenjit and Sumathi, 2005) so the response of microorganisms towards toxic heavy metals is of importance in view of the interest in the reclamation of polluted sites (Shankar et al., 2007). Microorganisms uptake metals either actively (bioaccumulation) and/or passively (biosorption) (Shumate and Strandberg, 1985; Anders and Hubert, 1992; Hussein et al., 2004). Bioaccumulation is the active method of metal accumulation by living cells. The capacity of living cells to remove metal ions from environment is influenced by environmental growth conditions, as temperature, pH and biomass concentrations (Abd-El-Raheem et al., 2013).

TEM is a useful technique that can help to localize and to identify metals deposited within or around microbial cells. Identification of the site of accumulation is important as it can give clues to the biochemical mechanisms driving metal accumulation. Biological materials which are largely composed of light elements such as C, N, H, O, P, and S, do not deflect the electron beam to the same degree. Thus, it is possible to visualize metals against the faint image of a bacterial cell (Lloyd and Macaskie, 2002).

The present study, aims at isolating *S. oneindensis* from Basra soil, south of Iraq, and evaluating metals bioaccumulation ability, and also studying the effect of metals initial concentration, contact times, and determine the cellular localization of accumulated metals within this bacterium by using Transmission electron microscope.

MATERIALS AND METHODS

Isolation of bacteria

Three soil samples (30 g each) were collected from AL-Zubair district west of Basra city- Iraq during January 2013. The samples were collected using a sterile plastic bag and transferred within 2 h to laboratory for analysis. One gram of air dried soil sample was serially diluted using sterilized distilled water and spread over nutrient agar. The plates were incubated at 30°C for 24 h.

Bacterial characterization

Properties of the bacteria included gram stain, citrate utilization, indole production, methyl red, nitrate reduction, Voges Proskauer, catalase, dextrose, mannitol and sucrose utilization, starch hydrolysis, and gelatin liquefaction tests were determined according to Sneath et al. (1986).

S16 rRNA gene based identification

The isolates were identified by sequencing of the 16S rRNA gene. To determine the identification of bacterial isolates, the amplified 16S rRNA gene PCR products obtained from total genomic DNA using primer set 27F (5′ AGAGTTTGATCCTGGCTCAG-3′) and 72.1492R (5′ GGTTACCTTGTTACGACTT-3′), (Lane et al., 1985) were sequenced commercially. DNA sequences obtained were compared to sequences available online in a GenBank database (http://www.ncbi.nlm.nih.gov). Homology search was performed using Bioinformatics tools available online BLASTn www.ncbi.nlm.nih.gov/BLA (Altschul et al., 1997).

Determination of minimal inhibitory concentrations (MIC) for Pb and Cd

The minimum inhibitory concentration (MIC) of Cd and Pb of bacteria were determined by disc diffusion method. The concentrations of Cd and Pb were between 40 to 2500 mgl^{-1}. Filter paper discs were saturated with heavy metals for 30 min, and then placed on nutrient agar plates and incubated for 24 h at 30°C. Pb $(NO_3)_2$ and $CdCl_2$ were used to prepare mother solution of these metals in sterile distilled water and were used in various concentrations. The lowest concentrations of Cd and Pb that completely prevented growth of each bacterium were considered as the MIC (Sethuraman and Kumar, 2011).

Bioaccumulation of heavy metals by bacteria

Bacteria were grown in LB broth containing 5, 10, 25 and 50 mgl^{-1} of lead and for cadmium 10, 20, 50 and 100 mgl^{-1} then incubated for 2, 4, 6, 24 and 48 h at 30°C in a shaker incubator at 150 rpm. Three replicates for each concentration have been done, and one as a control. The bacterial cells were harvested by centrifugation at 6000 rpm for 15 min, and suspended in 1 ml of distilled water, oven-dried at 80°C for 1 h and weighted. Metal concentrations were measured by atomic absorption spectrophotometer. Control was represented by the same microbial culture without heavy metals. Each metals concentration is measured with two replicates (Sprocati et al., 2006).

Transmission electron microscope

By centrifuging samples broth culture for 10 min at 3000 rpm, and decanting the supernatant, fixing pellet with 4% gutaraldehyde for 4 h at 4°C and centrifuged again, decanting fixative and adding an appropriate quantity animal serum to submerge sample, and allowing serum to clot. It was washed three times with 0.1 M Cacodylate buffer for 10 min. and Posted fix in 1% Osmium tetrroxide for 2 h at 4°C. Also, it was washed again three times with 0.1 M Cacodylate buffer for 10 min. Dehydrating in series of

Table 1. Biochemical characteristics of *S. oneidensis* isolate from soils.

Tests	Characteristics observed
Oxidase test	+
Catalase test	+
Indole formation	-
Nitrate reduction	-
Production of H_2S	+
Gelatin liquefaction	+
Fermentation of	
Sucrose	+
Fructose	**+**
D-glucose	+

"+"and "-" indicate positive and negative reactions, respectively.

acetone of 35, 50, 75, 95, and 100% for 10, 10,10, 10 and 15 min respectively. Finally, we make infiltration of the specimen with acetone and resin:

Acetone:	Resin	Time
1	: 1	1 h
1	: 3	2 h
100%		Overnight
100%		2 h

Embedding: Specimens were placed into beam capsule filled with resin. Polymerization: polymerized in oven at 60°C for 24 h. Make ultrasectioning, by choosing an area of interest, then cut for ultrathin section, selecting the silver section, picking up a section with a grid, then drying with filter paper. Finally staining with Uranyl acetate for 15 min, and washed double distills water. Lead stained for 10 min, and washed twice in distilled water. This work was done at the Electron Microscope Laboratory Institute of Bioscience, University Putra Malaysia.

RESULTS AND DISCUSSION

Characterization and molecular identification of isolated bacteria

The selected bacterium was characterized and identified by using conventional morphological, physiological and biochemical tests (Table 1). It was presumptively identified as *Shewanella* sp (Holt et al., 2005). The sequence of 16S rRNA gene of this bacterium was submitted to Blastn database 16S ribosomal RNA sequences (Bacteria and Archaea) Megablast http://www.ncbinlm.nih.gov/blast. It indicated a close genetic relatedness of this bacterium with the rDNA sequence of *Shewanella oneidensis* (Holt et al., 2005).

Minimum inhibitory concentration (MIC)

The MIC is the lowest concentration of the heavy metals that completely inhibited bacterial growth (Froidevaux et al., 2001) *S. oneindensis* showed significant resistance to high concentrations of Pb and Cd (700 and 1000 mgl^{-1}) respectively. This may be considered new finding, that the other studies showed different results. Chihomvu et al. (2014) recorded MIC for Pb by *Shewanella* (4 Mm), while MIC was 0 for Cd. Francis and Dodge (1988) and Toes et al. (2008) demonstrated that, the tolerances inhibited growth of different *Shewanella* strains completely at 150 µM Co, 150 - 400 µM Zn, 75 - 150 µM Cd, and 150 µM Cu when cultivated aerobically in 10% LB broth. The effect of the medium on metal toxicity was demonstrated in a study by Toes et al. (2008) where higher tolerances of Cu by *Shewanella* between 75 and 750 µM in more nutrient rich media and the presence of manganese oxides also reduce the toxicity of Cu.

Bioaccumulation

S. oneidensis as sulfate reducing bacteria has the potential to enhance metal retention via extracellular binding, cellular uptake and accumulation of metals, oxidation/reduction processes, and surface mediated mineral precipitation (Burkhardt, 2010). From results of the present study, *S. oneindensis* was able to accumulate Cd than Pb (26.77 and 3.98 mgg^{-1}) at 48 h and at concentrations 50 and 100 mgl^{-1} respectively (Figures 1 and 2). The differences in this accumulation ability for these two metals may be related to different toxicity of these metals to this bacterium. From the results, the accumulation of both metals increases with increasing the time. Varghese et al. (2012) showed that, with increasing time, the biomass of the bacterial strains increased. Likewise, with an increase in biomass, the heavy metals bioaccumulation also increased. The results of the present study showed that the high amount of accumulation occurs with high metals concentration (50 and 100 mgl^{-1}). These results agree with the results reported by Odokuma and Akponah (2010), where they concluded an increasing uptake pattern observed in the respective test isolates as the initial concentration of the various heavy metal salts were increased. These observations suggested that metal uptake may involve diffusion phenomenon, whereby metal ions move from regions of high to low concentrations.

Transmission electron microscope

Cells were evaluated by TEM to observe the locations of precipitate of metals in relation to the *S. oneidensis* cells. In order to differentiate whether extracellular or intracellular reduction occurred, the cells were stained with uranyl acetate. Figure 3 has shown the cells before being exposed to the metals (a). Dark precipitate can be seen around the inside of the cell membrane, indicating

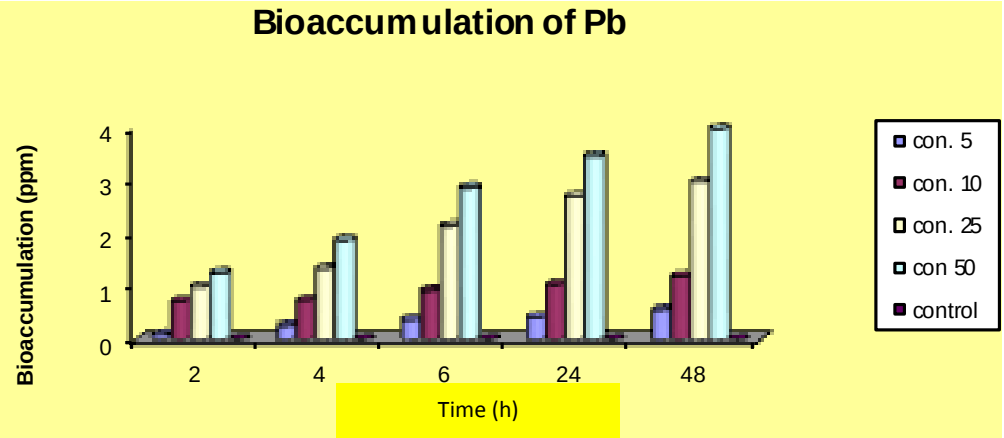

Figure 1. Bioaccumulation of Pb by *S. oneidensis* during different incubation periods and different concentrations.

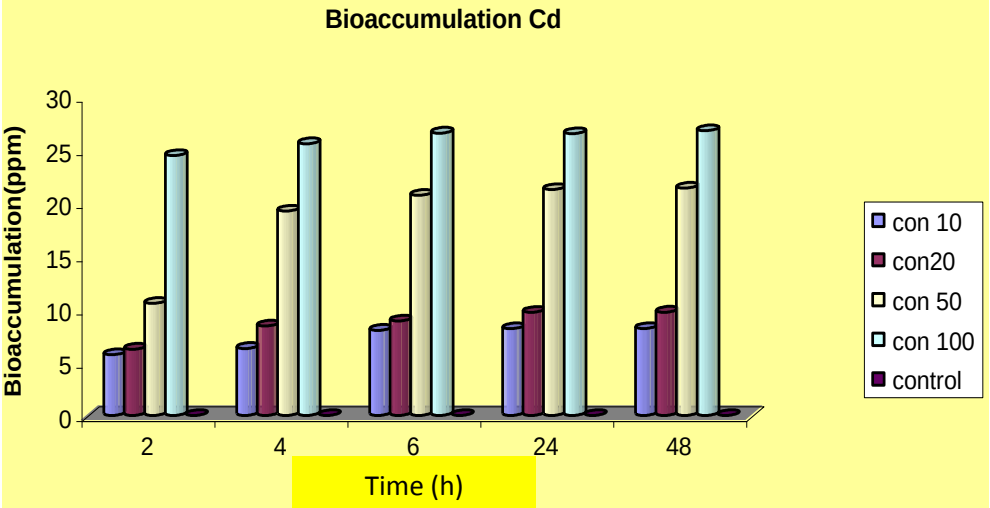

Figure 2. Bioaccumulation of Cd by *S. oneidensis* during different incubation periods and different concentrations.

intracellular Cd and Pb reduction has occurred (b and c). Also, from Figure (3b and c) there were changes in size and shape of cells and some cells have been lysed. These results could add to the toxicity of the substance, and ultimately results in cell death.

The cell surface morphology considerably changed after metals exposure. The cellular localization of the metals bound by the cells of the bacterium was located mainly within the cell membrane. However, some intracellular metal accumulates were also identified in the cytoplasm of the bacterial cells. Merroun et al. (2005) reported that, the cellular localization of the uranium bound by the cells of three types of *Acidithiobacillus ferrooxidans* was studied using TEM. Also, El-Helow et al. (2000) reported that, cell surfaces of cultures treated with cadmium chloride tended to be rough, suggesting that the cell increased its surface to improve the interaction of toxic substances with the cell surface. Also, Singh et al. (2013), reported cell surface morphological changes in *Cryptococcus* sp. after exposure to heavy metals, and which could be observed by the presence of shrunken and distorted cell wall in the presence of Cd and depressions in the presence of Pb and Zn.

Secretion of extracellular polymeric substance by *Desulfovibrio desulfuricans* during biosorption of Zn and Cu was reported to modify its cell surface morphology (Chen et al., 2000). Similarly, El-Meleigy et al. (2013) reported that, high dark dense cytoplasm due to Co^{2+} precipitation is partially emptied with a very thick cell wall; changing in the morphology of vegetative cells of *Bacillus firmus* and *Bacillus subtilis.*

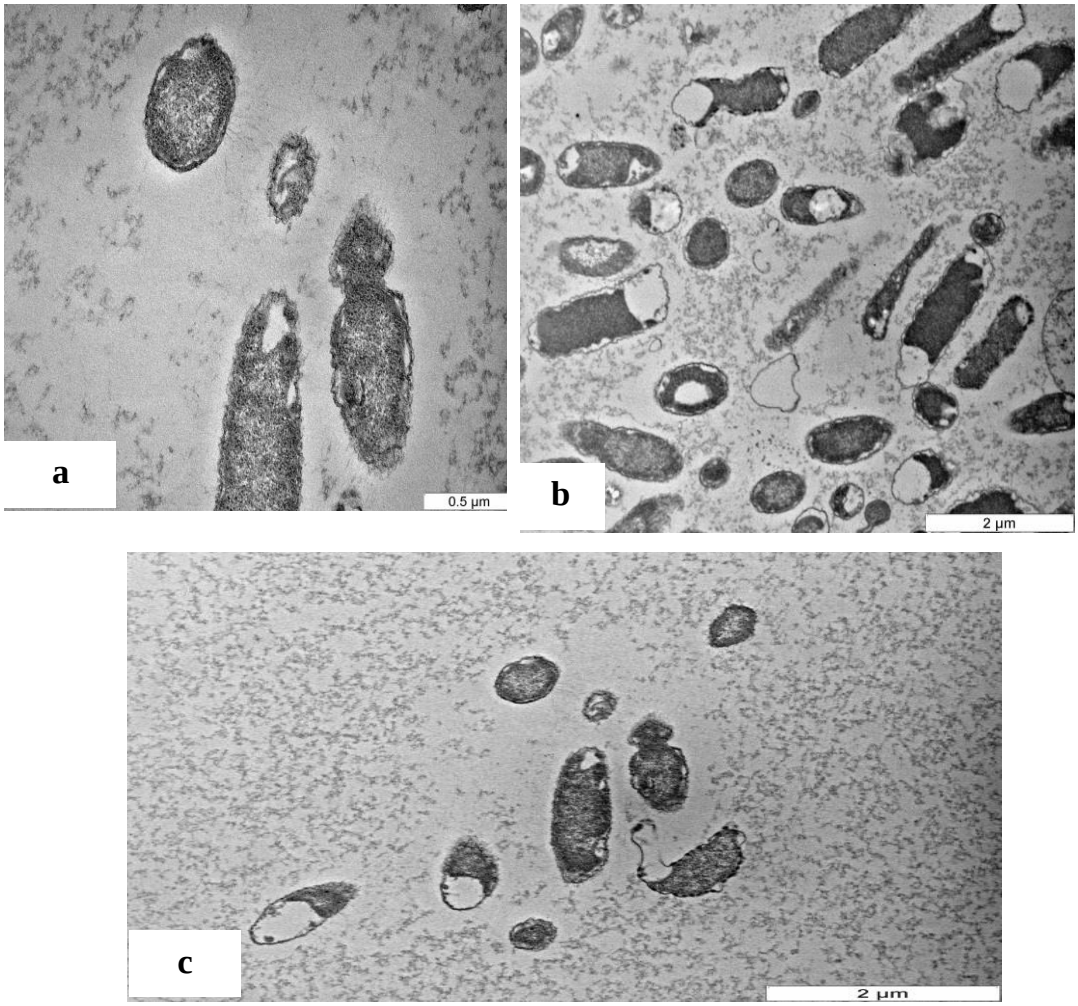

Figure 3. Transmission electron micrographs of *S. oneidensis* a: control, b: treated with 50 mgl^{-1} of Cd for 24 h, c: Treated with 50 mgl^{-1} of Pb for 24 h (Scale of bar 0.5 and 2 µm).

Conflict of interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Prevalence of exfoliative toxins and toxic shock syndrome toxin-1 encoding genes among coagulase positive *Staphylococcus* isolated from human and animal sources

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This study was undertaken to determine the prevalence of coagulase positive staphylococcus (CPS) in Jordan and to investigate the presence of genes encoding exfoliative toxins (*eta*, *etb*), and toxic shock syndrome toxin-1 (*tst*). Seven hundred and fifty three samples were used including 273 obtained from human sources and 480 from animals (sheep, cows, and goats). One hundred and sixty seven isolates were identified as CPS and appeared as gram positive cocci, non-motile, produced coagulase, catalase, reduce tellurite, were resistant to acriflavin, unable to produce oxidase and amylase. The prevalence of CPS colonizing human was 115(42.1%) with 26.0% in nasal and 16.0% in nails. Livestock-associated CPS was detected in 52(10.8%) of the samples. polymerase chain reaction (PCR) amplification revealed *eta* to be the most common toxin gene detected in 36.5 and 28.8%, followed by *tst* in 25.2 and 5.76% of human and animal isolates, respectively. The possession of various gene combinations was found in 15(8.98%) of the isolates including *eta* plus *tst* in 14(12.2%) and *eta* plus *etb* in 1(0.86%) of human isolates. Polymerase chain reaction -restriction fragment length polymorphism (PCR-RFLP) assay was performed for all CPS by using *TaqI* restriction; the pattern revealed that 163(97.6%) were *Staphylococcus aureus* and were identified phenotypically and confirmed genotypically by amplification of *kat* gene, and 4(2.40%) identified as *Staphylococcus pseudintermedius*. Phylogenetic analysis indicated that clones characterized in this study were *S. aureus* subsp. *aureus* and *S. pseudintermedius*. Toxins genes are mostly prevalent among *S. aureus* subsp. *aureus* strains.

Key words: Coagulase positive *Staphylococcus*, exfoliative toxins, toxic shock syndrome toxin-1

INTRODUCTION

Staphylococcus sp. are one of the most commonly found pathogenic bacteria in human environment. The epidemiology of *Staphylococcus* species in animals has gained interest in the last years, not only for their importance in veterinary medicine, but for its increasingly evidenced zoonotic potential. The genus includes both

human and animal pathogens, generally coagulase-positive staphylococci (CPS) such as *S. aureus*, *S. intermedius*, *S. delphini*, *S. hyicus*, *S. schleiferi* subsp. *coagulans*, and *S. pseudintermedius*, and coagulase negative staphylococci (CNS) such as *S. equorum*, *S. xylosus*, *S. saprophyticus*, *S. succinus*, *S. warneri*, *S. epidermidis*, and *S. lentus* (Devriese et al., 2008). *Staphylococcus aureus* is a dangerous human pathogen responsible for a wide variety of diseases. Other species are difficult to identify as frank human pathogens, but a few reports have described other coagulase positive staphylococci as causing opportunistic infections (Van Hoovels et al., 2006).

Nearly all *S. aureus* strains secrete exoproteins such as coagulase, nucleases, proteases, lipases, hyaluronidase and collagenase. Staphylocoagulase (SC) causes coagulation of plasma and is regarded as a marker for discriminating *S. aureus* from other less pathogenetic staphylococci. The nuclease enzyme is the major regulator of *S. aureus* virulence determinants (Olson et al., 2013) and the amplification of the *nuc* gene has a potential for the rapid diagnosis of *S. aureus* infections (Costa et al., 2005). Staphylococci secrete a wide spectrum of diverse extracellular proteins that are coordinately expressed during different stages of infection by a network of virulence regulators which render the bacterium virulent (Cotar et al., 2010). These include adhesion factors, toxic proteins/enzymes, and exotoxins including exfoliative (*eta*, and *etb*), staphylococcal enterotoxins (SEs), and toxic shock syndrome toxin-1 (TSST-1) (Nemati et al., 2013).

As major virulence factors of *S. aureus*, TSST-1, and ETs (A and B) are pyrogenic toxins that have been implicated in host colonization, invasion of damaged skin and mucus, and evasion of host defense mechanisms. They are responsible for specific acute staphylococcal toxemia syndromes (Udo et al., 2009). Exfoliative toxins (also known as “epidermolytic” toxins) are particularly interesting virulence factors of *S. aureus*. These extremely specific serine proteases recognize and cleave desmosomal cadherins only in the superficial layers of the skin, which is directly responsible for the clinical manifestation of staphylococcal scalded skin syndrome (SSSS) (Bukowski et al., 2010). However, a significant increasing rate of ETs was recorded for nasal and clinical isolates (Dağı et al., 2015). The production of *eta* and *etb* has been examined in *S. aureus* strains and other strains associated with SSSS (Plano, 2004). Other CPS species, such as *S. intermedius* and *S. hyicus*, produce similar toxins (Ahrens and Andresen, 2004). However, it has not been fully understood whether the toxins are produced only by human strains or whether animal strains also produce them. Direct contact between animals and humans is a relevant factor to take into account to understand the prevalence and the evolution of *Staphylococcus* species. As *S. aureus* could pass from livestock to humans, it could be public health problem.

Clinically, toxic shock syndrome is closely associated with strains of *S. aureus* carrying the gene encoding for *tst* and associated mostly with tampon use (McDermott and Sheridan, 2015). The gene encoding *tst* is a chromosomal, and the toxin is symptomatically related to the staphylococcal enterotoxin group of toxins which are included in the pyrogenic toxin superantigen families (PTSAgs). PTSAgs exert their virulence by binding to the major histocompatibility complex (MHC) class II molecules and the Vβ chain of the T-cell receptor (TCR) from the outside in a nonspecific manner. This leads to the stimulation of T-cell proliferation, the release of inflammatory cytokines, and ultimately the suppression of the host immune system (Larkin et al., 2009).

Due to the high sensitivity and specificity they provide, molecular markers are an alternative tool for accurate identification and classification of *Staphylococcus* species. Molecular assays targeting some genes such as *hsp60*, 16S rRNA gene, *femA*, *dnaJ* (Hauschild and Stepanovic, 2008), and catalase (*kat*) gene have been used for reliably identifying and classifying staphylococci (Blaiotta et al., 2010). The aim of the present study was to characterize CPS isolated from human and animal sources in Jordan and to investigate the prevalence of *ETs* and *tst* genes among the isolates. In addition, PCR-RFLP analysis of the *kat* gene was employed for a genotypic study.

MATERIALS AND METHODS

Collection of samples

A total of 753 samples were collected from human and animal sources during the period from October 2012 to May 2013.

Human sources

One hundred healthy students (53 female and 47 male) at Al-Balqa' Applied University were enrolled as volunteers. Two swabs were obtained from each student, one from nasal, and the other from nail. A written informed consent was obtained from all the volunteers in the study. Swabs were incubated on Tryptic Soy Broth (TSB) supplemented with 7% NaCl for 24 to 48 h at 37°C. In addition, 73 *S. aureus* isolates from routine microbiological specimen were collected from different hospitals in Jordan. The isolates were obtained from cultures of different specimens including blood infection, urinary tract infection, abscess, and septicemia. Suspected colonies of *S. aureus* from primary culture plates of Blood agar (BA), and Mannitol Salt Agar (MSA) were confirmed, by Gram reaction, positive catalase, tube coagulase and

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Table 1. Base sequences, locations within the genes, and predicted sizes of amplified products for the Staphylococcal toxin-specific oligonucleotide primers.

Gene	Primer	Oligonucleotide sequence (5'-3')	Location within genes	Size of amplified product (bp)
tst	TSST-1	atggcagcatcagcttgata	251-270	
	TSST-2	tttccaataaccacccgttt	581-600	350
eta	ETA-1	ctagtgcatttgttattcaa	374-393	
	ETA2	tgcattgacaccatagtact	473-492	119
etb	ETB-1	acggctatatacattcaatt	51-70	
	ETB-2	tccatcgataatatacctaa	231-250	200

Deoxyribonucleases (DNAse) test. Nasal, nail, and clinical isolates were subcultured on MSA and Baird Parker Agar (BPA), and incubated at 37°C for 24 to 48 h. The suspected colonies were maintained on Staphylococcal agar no.110 (Fabiano et al., 2008) and were kept at 4°C for identification (Abdul Aziz et al., 2013).

Animal sources

Four hundred and eighty samples were collected from three central slaughterhouses in Jordan which represent the population of animals in Amman city (capital of Jordan). Samples were imported meat (Romanian, Australian) from177 sheep, 47 goats, and 16 cows. The age of the animals ranged from 1.5 to 3 years. Two samples were obtained from each animal, one from nares before slaughtering and others from various muscle sites by collection of segments of the muscle or from swabs of slaughtered animals. Samples were enriched in TSB containing 7% NaCl for 24 to 48 h of incubation at 37°C (Isenberg, 2004). A portion of the enrichment cultures were then streaked on MSA and BPA and incubated at 37°C for 24 to 48 h. The suspected colonies were maintained on Staphylococcal 110 (SM1110) media and kept at 4°C for identification.

Isolation and biochemical characterization of CPS

S. aureus strain (ATCC 25923) was used as a positive control for all biochemical and molecular tests. Identification of CPS was done by using selective and differential agar media. Isolates were examined by Gram stain (Bremer et al., 2004), catalase (Benson, 2002), coagulase and clumping-factor tests were carried out for the detection of staphylocoagulase (bioMe´rieux) (Winn et al., 2006) using rabbit and human plasma, mannitol fermentation (Kateete et al., 2010), oxidase (Faller and Schleifer, 1981), hemolysin production using human RBCs (Abou-Elela et al., 2009), amylase (Mishra and Behera, 2008), protease (Cheesbrough, 2002), lipase (Edberg et al., 1996), sensitivity to acriflavin (Roberson et al., 1992), lysostaphin susceptibility (Schleifer et al., 1981), acetoin production (Chapin and Murray, 1999), lecithinase (Cotar et al., 2008), tellurite reduction (Andrews, 1992), thermostable nuclease (Pascual-Anderson, 1992) and DNase test (Sánchez et al., 2003).

Molecular characterization of CPS

Extraction of genomic DNA

Bacterial culture was grown overnight on nutrient broth; 2 ml of the culture was transferred into a microcentrifuge tube and spun at 5,000 x g for 5 min. The pellet was resuspended in 567 µl of Tris-EDTA (TE) buffer to which 30 µl of 10% SDS and 3 µl of 20 mg/ml proteinase K were added. The mixture was inverted gently and incubated for 1 h at 37°C. After incubation, 100 µl of 5M NaCl was added and mixed thoroughly. Then, eighty microliter of 10% Cetyl trimethylammonium bromide (CTAB) - 0.7 M NaCl solution was added and the tubes were incubated for further 10 min at 65°C. Equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added, mixed well and centrifuged at 10,000 rpm for 10 min. The upper aqueous phase was transferred to a new tube and an equal volume of chloroform/isoamyl alcohol (24:1) was added and centrifuged at 10,000 rpm for 10 min. The upper aqueous phase was then transferred to a new tube and 0.8 volume of isopropanol was added, mixed gently until the DNA was precipitated. The DNA was washed with 70% ethanol and resuspended in 50 µl TE buffer (Rallapalli et al., 2008).

Detection of tst, eta, and etb toxins genes by PCR

The primers used for detection of *tst, eta, and etb* genes are listed in Table 1 (Johnson et al., 1991). Each polymerase chain reaction (PCR) contained 2.5 µl of 10X PCR Buffer, 1.0 µM $MgCl_2$, 200 µM dNTP, 1U *Taq* DNA polymerase, 10 pmol of each primer, and 50 ng/ul of template DNA. The final volume was adjusted to 25 µl by adding sterile ultrapure water. DNA amplification was performed using the following amplification conditions: initial denaturation for 5 min at 94°C followed by 30 cycles of denaturation (94°C for 2 min), annealing (50°C for 1 min), and extension (72°C for 1 min). A final extension step (72°C for 5 min) was employed after the completion of the cycles (Rall et al., 2008).

Identification of CPS species by PCR-RFLP

Two oligonucleotide primers were used in this part: CPSK1F (CARAAYAACTGGGATTTCTGGAC) and CPSK6R (GCATCRCCRTAWGAGAATAAACG) from the highly conserved region of *S. aureus kat* gene sequences found in GenBank. Targeting positions were 487-509bp and 1031-1009 bp of the *kat* gene of *S. aureus* subsp. *aureus* Mu50 (BA000017) which allowed the amplification of 544 bp fragment. PCR amplifications were performed with a total volume of 50 µl, including: 5 µl of template DNA, 5 µl of 10X buffer PCR buffer, 2.5 µl of 50 mM $MgCl_2$, 0.5 µl of dNTP mix (25 mM of each dNTPs), 0.2 µl of each primer (0.1 mM), and 0.2 µl of *Taq* DNA polymerase solution (5 U/µl). PCR amplification conditions consisted of an initial denaturing step (95°C

Table 2. Distribution of animal samples according to their sources.

Imported meat (N=280)			Local meat (N=200)		
Type of samples	**Sheep**	**Cows**	**Sheep**	**Goats**	**Cows**
Meat pieces	76	14	8	42	0
Meat swabs	50	0	43	5	2
Nasal swabs	126	14	51	47	2
Total	252	28	102	94	4

Table 3. Numbers and percentages of coagulase positive *Staphylococcus* isolates collected from different sources.

Samples	Sources	No. of samples	No. and (%) of CPS	Total
Human (N=273)	Nasal swabs	100	26 (26%)	115
	Nail swabs	100	16 (16%)	
	Clinical samples	73	73 (100%)	
Animal (N=480)	Nasal swabs	240	13 (5.4%)	52
	Meat pieces	140	34 (24.2%)	
	Meat swabs	100	5 (5%)	

for 3 min) and 40 amplification cycles: a denaturing step for 10s at 95°C and an annealing-extension step for 45 sat 56°C. After amplification, 15 µl of each PCR mixtures were tested by electrophoresis on 1.5% (w/v) agarose gel at 100 V for 1 h. The remaining part (35 µl) of the PCR product was digested in a total volume of 50 µl by 20 U of TaqI restriction endonuclease at 65°C for 2 h. Restriction fragments were resolved by electrophoresis on 2% (w/v) agarose gel at 100V for 2 h (Blaiotta et al., 2010).

Identification of CPS species and subspecies by DNA sequencing

From the highly conserved region of *kat* gene sequences of CPS found in genbank two oligonucleotide primers were selected: CPSK1F7 (CARAAYAACTGGGATTTCTGGAC) and CPSK6R (GCATCRCCRTAWGAGAATAAACG) (Macrogen Inc. Seoul, South Korea). According to the variation in biochemical results and PCR-RFLP band patterns, strains were choice to sequence. Analysis of DNA similarity was performed using BLAST programs (Basic Local Alignment Search Tool).

Phylogenetic analysis

Using the keyword "catalase *Staphylococcus*", sequences of catalase genes from different *Staphylococcus* species and isolates were retrieved from the National Center for Biotechnology Information (NCBI) site (www.ncbi.nlm.nih.gov). A phylogenetic tree of catalase genes was constructed using Molecular Evolutionary Genetics Analysis (MEGA) version 5.2 (Tamura et al., 2011). The Neighbor-joining (NJ) method of tree generation was used to assess the evolutionary relationships (Saitou and Nei, 1987), and the significance of clustering was evaluated by bootstrapping with 1000 replications.

RESULTS

In this study, a total of 753 samples were obtained from 273 human (100 nasal swabs, 100 nail swabs, and 73 clinical samples), and 480 animal sources (240 nasal swabs, 240 meat (pieces and swabs). Samples distribution in respect to their livestock sources was shown in Table 2. According to colonies morphology and coagulase test, only 167 isolates were characterized as CPS (Table 3). The prevalence of CPS colonizing humans was 115 including: 26(26%) nasal swabs, 16(16%) nail swabs. CPS are present in seven cases in both the nail and the nasal of the same person. However, 52 of CPS was isolated from animal sources and distributed as follows: nasal swabs isolates was detected in 13(5.4%), meat pieces in 34(24.2%), and meat swabs in 5(5%) of the tested samples.

Characterization of coagulase positive *staphylococcus*

One hundered and sixty seven CPS isolates obtained from human and animal sources were characterized by different biochemical tests (Table 4). All of CPS isolates were able to grow on P agar supplemented with acriflavine, able to produce catalase, reduce tellurite, and did not produce oxidase or amylase enzymes. However, CPS isolated from human sources produced more virulance factors than animal isolates.

Molecular identification

The prevalence of toxin genes (eta, etb, and tst) in CPS isolates

The *eta, etb* and *tst* genes positive isolates produced

Table 4. Phenotypic characteristic of coagulase positive *Staphylococcus* isolated from human and animal sources.

Biochemical tests	Human sources (N=115)	Animal sources (N=52)
Gram stain	115 (100%)	52 (100%)
Catalase	115 (100%)	52 (100%)
Coagulase	115 (100%)	52 (100%)
Mannitol fermentation	115 (100%)	50 (96.1%)
Oxidase	0 (0%)	0 (0%)
Hemolysin	101 (87.8%)	39 (75%)
Amylase	0 (0%)	0 (0%)
Protease	109 (94.7%)	40 (76.9%)
Lipase	112 (97.39%)	41 (78.8%)
Lecithinase	96 (83.47%)	36 (69.2%)
Tellurite	115 (100%)	52 (100%)
Lysostaphin	115 (100%)	49 (94.2%)
DNase	112 (97.39%)	46 (88.4%)
TNase	112 (97.39%)	51 (98%)
Acetoin	110 (95.6%)	40 (76.9%)
Acriflavin sensitivity	115 (100%)	52 (100%)
Oxidation / Fermentation	115 (100%)	50 (96.1%)

Table 5. The prevalence of *eta, etb*, and *tst* genes among CPS isolated from human and animal sources.

Type of toxins	Human sources (N=115)			Animal sources (N= 52)	
	Nasal N=26	Nail N=16	Clinical N=73	Nasal N=13	Meat N=39
Exofoliative A (*eta*)	6 (23%)	0 (0%)	36 (49.3%)	0 (0%)	15 (38.4%)
Exofoliative B (*etb*)	2 (7.6%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
TST (*tst*)	10 (38.4%)	0 (0%)	19 (26%)	3 (23%)	0 (0%)
Exofoliative A+ Exofoliative B	1 (3.8%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Exofoliative A+ TST	4 (15.4%)	0 (0%)	10 (13.7%)	0 (0%)	0 (0%)

119, 200 and 350 bp, respectively. The gene coding for *eta* toxin was the most frequent among isolates obtained from human sources which showed 23 and 49.3% for nasal and clinical., respectively. However, 38.4% of meat isolates showed positive results for the *eta* toxin. On the other hand, only 7.6% of humans expressed *etb* gene in the noses. The possession of various genes combination was found in 15 (8.98%) of all the isolates obtained from human sources. Nasal (15.38%) and clinical (13.7%) showed *eta* plus *tst*, whereas, (3.8%) of nasal isolates showed *eta* plus *etb* genes (Table 5). The prevalence of *eta, etb,* and *tst* among human isolates are more than animal isolates.

Differentiation of CPS species by PCR-RFLP approach

A fragment of 544 bp for *catalase* gene was amplified from 167 isolates. A clear differentiation at the species and subspecies levels was achieved by using PCR-RFLP analysis that was performed using PCR products from all isolates (Figure 1). Accordingly, 163 of the isolates were identified as *S. aureus* subsp. *aureus*, and 4 were identified as *S. pseudintermedius.* However, returning to the source of CPS, all S*. pseudintermedius* isolates were obtained from animal sources including 3 obtained from sheep and one from goats.

Phenotypic characterization revealed that *Staphylococcus aureus* subsp. *aureus* produced more virulence factors comparing with *S. pseudintermedius.* This is demonstrated by the high percentages of beta-hemolysin produced on blood agar supplemented with 5% (v/v) human blood, lipase, lecithinase, acetoin production, DNase and Thermonuclease activity (TNase). However, all *S. pseudintermedius* isolates were able to ferment mannitol and produce protease, alpha hemolysin and lysostaphin susceptibility, but unable to produce lecithinase enzyme and β- hemolysin. Toxigenicity study revealed that all of the exotoxin-producing isolates were

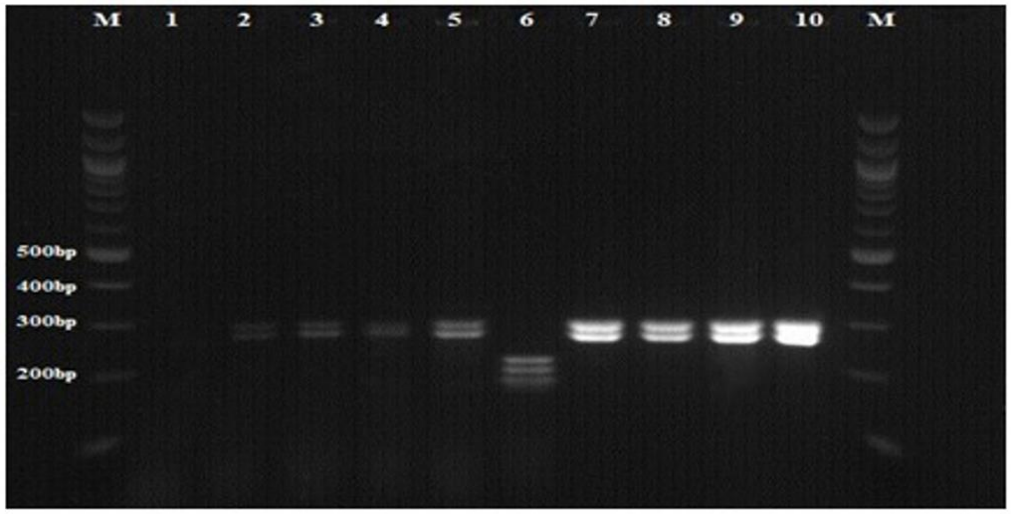

Figure 1. Agarose gel electrophoresis (2%) showed the *TaqI* RFLP analysis of Kat gene. Lane M, 1.25 Kb DNA marker; lane 1, negative control from CNS isolates, lane 2, PCR product for *S. aureus* isolated from human nail; lane 3, PCR product for *S. aureus* isolated from animal nose; lanes 4-5, PCR products for *S. aureus* isolated from meat; lanes 7-9, PCR products for *S. aureus* isolated from clinical sources, lane 6, product for *S. pseudintermedius* isolate from meat source, and lane 10, positive control (*S. aureus* ATCC 25923).

Table 6. Differences in phenotypic and genotypic characteristics produced by *S. aureus*, and *S. pseudintermedius* isolated from human and animal sources.

Biochemical tests	*S. aureus* (N=163)	*S. pseudintermedius* (N=4)
Coagulase	161(100%)	4 (100%)
Clumping factor	161 (100%)	0 (0%)
Mannitol utilization	161 (98.7%)	4 (100%)
Beta-hemolysis	138 (84.6%)	0 (0%)
Alpha-hemolysis	0 (0%)	2 (50%)
Protease	145 (88.9%)	4 (100%)
Lipase	152 (93.2%)	1 (25%)
Lecithinase	132 (80.9%)	0 (0%)
Lysostaphin	160 (98.1%)	4 (100%)
DNase	155 (95%)	3 (75%)
TNase	160 (98.1%)	3 (75%)
Acetoin production	148 (90.8%)	2 (50%)
Anaerobic fermentation	161 (98.7%)	4 (100%)
Exofoliative A	56 (34.3%)	1 (25.0%)
Exofoliative B	2 (1.22%)	0 (0%)
TST	32 (19.6%)	0 (0%)

belong to *S. aureus,* while only one isolate among four of *S. pseudintermedius* was able to produce *eta* toxin (Table 6).

Sequencing and bioinformatics analysis

For bioinformatics analysis to confirm the PCR-RFLP results, a set of PCR products representing different species was used for sequencing. The BLAST analysis of the sequencing results classified some isolates as *S. aureus* subsp. *aureus* and others as *S. pseudintermedius*. To determine the phylogenetic relationship of *catalase* sequences with the sequences of different *Staphylococcus* species and isolates, a bootstrap phylogenetic tree was constructed using the

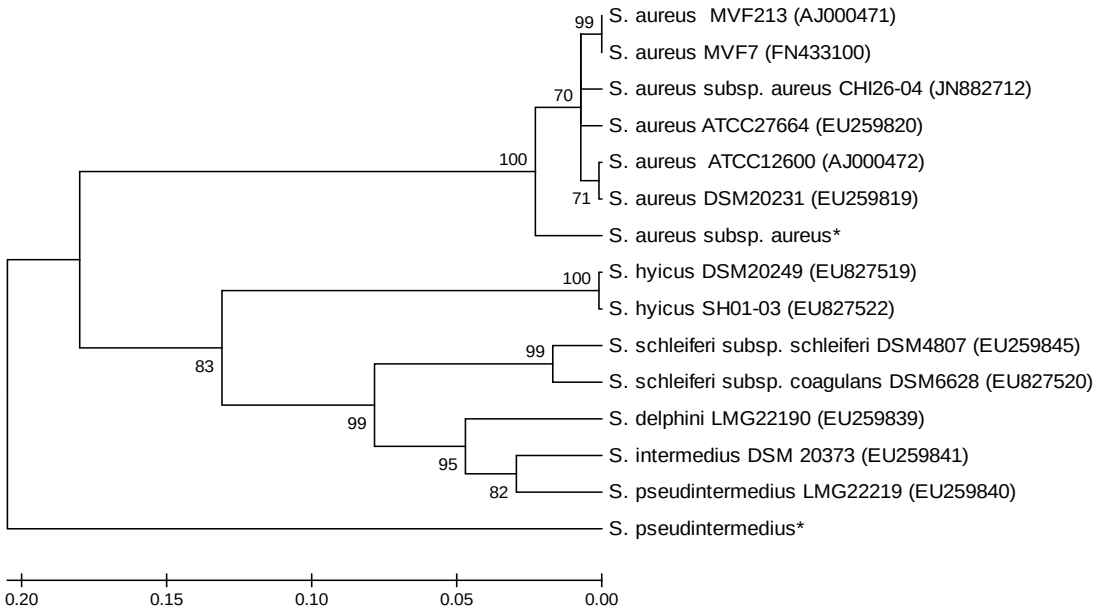

Figure 2. A phylogenetic tree based on the alignment of nucleotide sequences of *kat* gene from *Staphylococcus* species. The tree was constructed using the neighbor joining method provided in the MEGA 5.2 software. Numbers at each branch indicate the percentage of times a node was supported in 1,000 bootstrap pseudoreplication. The scale bar evaluates the sequence divergence. GenBank accession numbers are shown in parentheses and asterisks indicate sequences identified during this study.

neighbor joining method. The phylogenetic analysis clustered together the sequences of the same species (Figure 2). However, *S. pseudintermedius* identified in our study does not seems to be clustered with the other members of the intermedius group. Our findings confirms that the isolates characterized in this study represent *S. aureus* subsp*. aureus* and *S. pseudintermedius*.

DISCUSSION

S. aureus is one of the most commonly found pathogenic bacteria responsible for a broad range of nosocomial and community acquired infections due to an impressive array of toxins and other virulence determinants (Plata et al., 2009). It colonizes the skin and mucosa of human and several animal species. Although multiple body sites can be colonized in human beings, the anterior nares of the nose is the most frequent carriage site for *S. aureus* (Wertheim et al., 2005a). Extra-nasal sites that typically harbor the organism include the skin, perineum, and nails (Wertheim et al., 2005b). Accurate and rapid detection is important not only for choosing appropriate antibiotic therapy for the individual patient, but also for control of the endemicity of *S. aureus* infection.

The pathogenicity of *Staphylococcus* is related to the production of many virulence factors from which coagulase was considered as the most important one. In the present study, 26% of CPS was isolated from human nasal and 16% from nail samples. These results are in agreement with results obtained by other researchers (Al-Zahrani, 2012; Walther et al., 2012). However, Cross-sectional surveys of healthy adults populations have reported *S. aureus* nasal carriage rate of approximately 27% since 2000 (Wertheim et al., 2005a; Bischoff et al., 2004). This rate is much lower than the earlier reported prevalence of 35% which included studies since 1934 (Kluytmans et al., 1997). Improved personal hygiene and changes in socioeconomic class might explained this decline.

Strains present in the nose often contaminate the back of hands, fingers and face and so, nasal carriers can easily become skin carriers (Al-Zahrani, 2012). However, the prevalence of CPS among animal samples differed according to the sites of isolation. Nasal prevalence showed 5.4%, while meats give 16.25% (24.2 and 5% for pieces and swabs, respectively). These results were similar to the data from others (Abd El-Hamid and Bendary, 2013; Goja et al., 2013). Phenotypic characterization of CPS to species level was achieved applying growth on media supplemented with acriflavine, oxidase, mannitol utilization, hemolysin production, acetoin production, and amylase activity. However, 163 (97.6%) of CPS were characterized as typical *S. aureus*, while 4 (2.39%) of CPS were biochemically atypical by their production of α- hemolysin, the absence of clumping factor, and lecithinase production. The differences could be due to the diversity in the origin of the isolates (mainly animals), or might be due to some mutations that occur in the genes thus affecting the metabolic activity of the

species. In addition to the fact that most phenotypic identification systems have been developed for human health care and validated using clinical isolates obtained from human infections and thus might misclassify isolates from livestock (Zadoks and Watts, 2008).

Staphylococcus aureus can cause localized and invasive infections in humans. This is attributed to its ability to produce a variety of enzymes and toxins. Whereas nearly all strains of *S. aureus* produce enzymes that contribute to their pathogenicity, it has been generally accepted that only some strains produce ETs and PTSAgs (Bohach and Foster, 2000). In this study, the toxins genotypes of CPS were demonstrated. From one hundred and fifteen CPS isolates obtained from human source, 38.4% nasal and 26% clinical isolates possessed the gene for *tst.* These results are in accordance with previous findings that many healthy individuals are carriers of *tst*-producing strains of *S. aureus* (Mehrotra et al., 2000). The isolation of *S. aureus* strains possessing one of the pyrogenic toxins genes was previously described (Bawadi et al., 2009). In addition, half of the clinical isolates (49.3%) harbored the eta gene in comparison to (23%) for nasal. The notable higher prevalence of *tst* gene among clinical isolates indicates that the possession of this gene in particular seems to be a habitual feature of *S. aureus*. The resulted percentages are agreeable with the earlier reports and could be correlated with the transfer of this gene at high frequency (Moore and Lindsay, 2001).

On the other hand, only two human isolates harbored *etb* toxins genes in the nose (7.6%) in comparison to (0%) for other isolates. However, Becker et al. (2003) found that none of the clinical isolates were *etb* positive, while 1% of the nasal isolates were *etb* positive. Others also reported the absence of the genes encoding *etb* in the clinical isolates (Abd El-Hamid and Bendary, 2013). A geographic variation in the prevalence of different ETs isoform was reported. The majority of these reports confirmed that *eta* was the predominant ETs isoform in Europe, North America, and Africa which was similar to findings of this study, whereas *etb*-producing isolates were shown to be more frequent in Japan (Nishifuji et al., 2008). Screening for etb gene in larger samples is necessary to give better results concerning their prevalence's in different population. The possession of more than one toxin gene was found in 8.98% of human (clinical and nasal) isolates. However, *eta* plus *tst* and *eta* plus *etb* was found in 12.17 and 0.86%, respectively. Similar coexisting pyrogenic genes combination was reported by others (Becker et al., 2003). The contribution of pyrogenic genes combination to the overall pathogenicity potential of CPS should be investigated further.

The current study revealed that the gene coding for *eta* toxin, was the most frequent among human than animal isolates followed by *tst* gene. The higher prevalence of the *eta* gene in staphylococci could be explained by its greater immunogenicity (Yamasaki et al., 2005). However, the absence of *etb* toxin gene among animal isolates of CPS indicates that the gene cannot be held responsible for the diseases that may be induced by in animal and human. Strains that expressed *eta* and *tst* genes might form an alert for public health if they pass from poultry to human. A recent study by Nemati et al. (2013) reported the absence of ETs and *tst* genes in *S. aureus* isolated from animals. However, others reported the rare prevalence of exfoliative toxins among *S. aureus* isolates from animals (Endo et al., 2003). This indicates that these genes cannot be held responsible for the zoonotic diseases that may be induced in human. Moreover, Adesiyun et al. (1991) reported that ETs genes were observed in 3.9% of the examined animal's origin isolates. The present study confirms the relatively low prevalence of *eta*, *tst* encoded by genes in CPS isolated from animals and reported by others (Nemati et al., 2013). Although the importance of *tst* on animal health was not explained completely, it may play a role in the pathological mechanisms of bovine mastitis with its superantigenic functions (Zschock et al., 2000). Therefore, large-scale studies are required to determine the presence and role of *tst* in *S. aureus* isolates originating from livestock.

The accuracy of conventional methods for species identification and taxonomic classification of staphylococci based on phenotypic characteristics is limited (reported to be range from 50 to 70%) (Kloos and Bannerman, 1995). The use of nucleic acid targets, with their high sensitivity and specificity, provides an alternative technique for the accurate identification and classification of *Staphylococcus* species. Earlier results have been obtained by comparing sequences of certain genes such as *hsp60*, *sodA*, *rpoB*, *tuf*, and *gap* (Ghebremedhin et al., 2008). Blaiotta et al. (2010) evaluated the catalase (*kat*) gene performance as a new target for phylogenetic analysis of staphylococci and identification at the species level. The *kat* genes display a high level of restriction endonuclease polymorphism, offering good opportunities for rapid, and accurate species-level identification of staphylococcal isolates. All CPS isolates that have been identified phenotypically were confirmed genotypically by amplification of *kat* genes. In this study, the catalase gene of all CPS isolates was amplified by universal primers, allowing the amplification of a 544-bp region of *kat* containing polymorphic TaqI restriction sites of the various CPS isolates. Based on their PCR-RFLP patterns, 163 isolates were identified as *S. aureus subspecies aureus*, and only four isolates were identified as *S. pseudintermedius.* The *cat* gene sequence that determined in this study was similar to that already described by others (Blaiotta et al., 2010). To confirm the identification, some strains reclassified on the basis of their *TaqI* PCR-RFLP patterns were subjected to sequencing of the *kat* gene (544-bp fragment). The comparison of resulting sequences with

those from reference strains indicated an agreement with those of the PCR-RFLP analysis (*S. aureus subspecies aureus* showed 99% homology, while *S. pseudintermedius* showed 82% homology to the references). It seems that the *S. pseudintermedius* identified in this study does not clustered with the other members of the intermedius group. *S. pseudintermedius* colonization is uncommon in humans, even among people with frequent contact with animals (Talan et al., 1989). They are also rare among CPS isolates from hospitalized humans (Mahoudeau et al., 1997). Their importance as a zoonotic pathogen is therefore much smaller than that of other species. However, several cases of zoonotic transmission between companion animals and humans have been reported. In some cases humans were only colonized or contaminated, but in other cases transmission resulted in human infections (Guardabassi et al., 2004).

Despites the fact that the main host of *S. pseudintermedius* is the dogs and cats (Moodley et al., 2014), the results of this pilot study revealed that the four strains of *S. pseudintermedius* were isolated from livestock including 3(5.76%) from sheep and 1(1.92%) from goats. To our knowledge, this is the first report of *S. pseudintermedius* strains originating from sheep and goats samples worldwide. Although, Vasil (2007) reported the isolation of *S. intermedius* from sheep milk samples there is a high possibility, that these isolates were *S. pseudintermedius* and not *S. intermedius* since he depends on the biochemical tests only for identification. Direct contact between animals-animals and animals-humans is a relevant factor to take into account in understanding the epidemiology and evolution of this species. Van Hoovels et al. (2006) reported the first case of *S. pseudintermedius* infection in a human. However, Sasaki et al. (2007) also identified two strains from humans as being *S. pseudintermedius* strains.

Identification of Staphylococci to species level in microbiology is important to inform therapeutic intervention and management (Geraghty et al., 2013). In human, *S. pseudintermedius* is an opportunistic pathogen and a leading cause of skin and ear infections, post-operative wound infections in animals mainly the dogs and cats (Weese and van Duijkeren, 2010). This species has been recognized on a few occasions as a pathogen of rhinosinusitis, a catheter-related bacteremia, and an implantable cardioverter-defibrillator infections (Stegmann et al., 2010; Chuang, 2010). However, veterinary dermatologists and small animal clinical staff are sometimes considered as nasal carriers of *S. pseudintermedius* (Morris et al., 2010). The spectrum of *S. pseudintermedius* diseases have been expands which emphasizes the risk of zoonoses mainly in imunocompromised subjects (Savini et al., 2013).

Although, a limited knowledge concerning their pathogenecity was published, various virulence factors are known to be produced by this bacterium, (Fitzgerald, 2009) with the ETs as a major virulence factor (Iyori et al., 2010). This was demonstrated by the presence of 25% of the *S. pseudintermedius* strains that harbored *eta* toxin gene. By highlighting the virulence properties of the investigated strains, it has been found that all expressed high levels of protease, mannitol utilization, capable of anaerobic fermentation, and lysostaphin sensitivity. However, it is differed from *S. aureus* subsp*. aureus* by the absence of clumping-factor, and by their partial hemolytic activity (produced α- hemolysin by two strains). Unexpectedly, and although initially describing *S. pseudintermedius* as β -haemolytic, Awji et al. (2012) instead stated that the organism can be presumptively differentiated from *S. aureus* as the former lacks beta-haemolysis (using sheep blood agar). However, accurate phenotype observation remains crucial to reaching a conclusive bacterial diagnosis (Savini, 2013). Accordingly, the diagnostic algorithm of CPS should be reconsidered. It is likely that human and veterinary *S. pseudintermedius* isolates have been misidentified as *S. aureus*, *S. intermedius*, or other species (Bond and Loeffler, 2012). This fact should be considered when a patients' history includes contact with animals, the potential role of *S. pseudintermedius* as the agent of zoonoses has to be taken into account, and a correct identification may be performed. The isolation of livestock associated *S. pseudintermedius* in this pilot study could under line their possibility as a risk factor participating in human infections and emphasizing the need for correct species identification in clinical laboratories that handle samples of both human and animal origin. However, more useful genome-based investigations such as matrix-assisted laser desorption ionization-time of flight mass spectrometry could be used for profiling of staphylococcal strains using a large collection of staphylococci of diverse origins (David et al., 2010).

Although Livestock-associated and human-associated strains shared some virulence factors, but distinct virulence factors appeared to be important in host adaptation. Exchange of genes encoding these virulence factors between strains may expand the host range and thereby threaten public health (Fluit, 2012). More studies should be done to characterized animal isolates of CPS and prevent transferring species to health care settings. In conclusion, *S. aureus subspecies aureus* isolated from human seems to be different phenotypically and genotypically from livestock isolates.

Conflict of interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Growing varieties durum wheat (*Triticum durum*) in response to the effect of osmolytes and inoculation by *Azotobacter chroococcum* under salt stress

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This study was conducted to determine the effect of plant growth promoting rhizobacteria *Azotobacter chroococcum* AZ6 isolated from arid soil and osmolytes such as glycine betaine (GB) or proline (P) on the growth of durum wheat varieties under salinity stress. Inoculation by *A. chroococcum* AZ6 in the presence or absence of P (5 mM) or GB (5 mM) reduced substantially the effect of salt stress on plant growth parameters such as root length, plant height, fresh shoot and root weight and dry shoot and root weight. The differences between the two varieties were low but with a fresh and dry weight higher in Waha. The rate of Na^+ accumulation in the roots and the shoots was important up to 100 mM and increased at 200 mM. The K^+ concentration and chlorophyll content decreased but proline and amino acid contents were enhanced with increasing salinity. Treatment by inoculation in the presence or absence of osmolytes improved the chlorophyll (*a* and total) and the K^+ concentrations and reduced intracellular proline accumulation and amino acids contents. Also, as result, the use of *A. chroococcum* AZ6 and osmolytes treatment may provide a means of improving tolerance of durum wheat to salt stress.

Key words: Durum wheat, salinity, osmolytes, *Azotobacter chroococcum*,

INTRODUCTION

Salinity and aridity are major environmental constraints limiting the growth and productivity of crops. In arid and semi-arid areas of the world, rainfall is inadequate for the leaching of salts from the root zone. Accordingly, the soluble salts are accumulated in the soil surface with the Na^+ as a dominant cation. Soil salinity presents a growing threat to agriculture and causes salinization of arable land on the planet. One third of arable land resources in the world are affected by salinity (Munns, 2002). Losses in crop yields in saline areas are important. High

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concentrations of salts cause ion imbalance and hyper-osmotic stress in plants leading to cellular dehydration. The osmotic potential resulting from high concentrations of the Na^+ in the soil prevents the absorption of water which causes a variety of structural, biochemical and physiological seed changes and reduces the rate of germination causing a delay in the development of the plant (Poljakoff-Mayeber et al., 1994). Several experiments have been attempted to reduce the drastic effect of salt stress in the growth and productivity of plants. Most work focuses on the development of salt-resistant varieties. Thus, efforts to reproduce genotypes which are highly salt tolerant are hampered by a lack of understanding the complex nature of tolerance. Plant tolerance seems to be based on a number of mechanisms, many of which are based on physiological processes such as transport mechanisms that reduce or eliminate the Na^+ and the Cl^- of xylem (Tester and Davenport, 2003). Indeed, these varieties have developed various biochemical and physiological mechanisms against this type of stress. Such mechanism, ubiquitous in plants, is the accumulation of some organic metabolites of low molecular weight, especially during germination and early growth (Turan et al., 2007).

Exogenous application of osmolytes such as proline and glycine betaine attracted the attention of many researchers for several years (Wani et al., 2013). Thus, the application of low concentrations of glycine betaine and proline maintains a high concentration of the K^+ and activates the exclusion of the Na^+ and Cl^- ions from the leaves and roots (Cuin and Shabala, 2005).

Another biological approach exists. It is the plant growth promoting rhizobacteria (PGPR). It consists of inoculation of plants by rhizobacteria to promote their growth. PGPR used as biofertilizers and/or antagonists against plant pathogens are a promising alternative to chemical fertilizers and pesticides. Under salt stress conditions, PGPR have shown positive effects on plants, especially on physiological parameters such as germination, tolerance to drought, the weight of stems and roots (Tiwari et al., 2011). These rhizobacteria are able to adapt to adverse conditions and improve plant growth in environments at high osmolarity (Biari et al., 2008) and they develop molecular mechanisms to survive and grow with the increase of salinity (Tripathi et al., 2002).

According to many authors, most salt-tolerant bacteria accumulate or synthesize organic compatible solutes such as proline, glycine betaine and betaine glutamine. The application of compatible compounds and inoculation with rhizobacteria could attenuate the negative effects of salt stress on the growth of wheat.

Through this study, we evaluated the effects of inoculation by *Azotobacter chroococcum* AZ6 isolated from arid soil and exogenous application of glycine betaine (GB) and proline (P) on the morpho-biochemical parameters and on the ionic balance in two varieties of durum wheat under salt stress.

MATERIALS AND METHODS

Bacterial strain isolation

Soil samples were collected from the rhizosphere of wheat in an arid soil located in the region of Sétif situated at 300 km East of Algiers (Algeria). One gram of soil strongly adhering to the roots was extracted from the sample, added to 10 ml of sterile distilled water and shaken for 30 min. To select *Azotobacter*, the isolation was carried out on Ashby medium (Atlas, 2005), incubated at 28°C ± 1/72 h. Typical colonies were subcultured several times on Ashby agar to obtain pure cultures.

Identification of the bacterial strain

Among the isolated strains, AZ6 was identified according to macroscopic appearance (appearance of the colony on solid medium, form, texture and pigmentation), Gram staining, mobility, presence of cysts followed by preliminary biochemical characterization such as catalase test, oxidase and carbohydrate assimilation test (malonate, rhamnose and mannitol) according to Brenner et al.(2005).

16S rRNA gene sequence analysis

PCR fragments obtained by the amplification of a DNA fragment corresponding to a region of the 16S rDNA gene of the isolate were sequenced using the automatic sequencer at DNA Vision Company (http:/www.dnavision.com). The sequence was submitted to the GenBank, and accession number was assigned KT339176. The partial 16S rDNA sequence of the isolate strain was compared with those available in the databases. The phylogenetic tree was constructed on the aligned datasets using the Neighbor-Joining method (Saitou and Nei, 1987). Phylogenetic analyses were conducted in MEGA5 (Tamura et al., 2011).

Plant material

Seeds of two varieties of durum wheat were used: Waha (*Triticum durum* Waha *lcv*) and Bousselam (*Triticum durum* Bousselam *lcv*). They were obtained from Institut Technique des Grandes Cultures (ITGC)-Sétif–Algeria.

Bacterial inoculation and growth conditions

The strain AZ6 was cultured at 28°C/48 h in Winogradsky broth (Atlas, 2005). The culture was centrifuged (12,000 rpm/10 min) and rinsed twice in Phosphate-buffered saline (PBS) to obtain a density of 10^8 bacteria ml^{-1}. The two varieties of seeds Waha and Bousselam were surface sterilized with sodium hypochlorite solution (2%) for 30 min and rinsed several times with sterile distilled water. Seeds germination was carried out in advance on Whatman paper grade N° 42, in Petri dishes containing 15 ml sterile distilled water at 20°C/48 h in dark. Inoculation was effected on the germinated seeds by an immersion in the bacterial suspension for 30 min. Uninoculated seeds (control) were immersed in sterile distilled water. Plastic pots (Φ=10 cm), of which internal surface was disinfected with 70% of ethanol and the pots were filled with 200 g of sand washed thoroughly with water and autoclaved (120°C/1 h) during three successive days. 20 ml of Hoagland solution at ½ were added in each pot. The pots were then divided into three groups and each group was divided into four subgroups. The three groups represented the concentrations of NaCl used (control, 100 and 200 mM). The four subgroups indicated

the type of treatment: indicator (uninoculed seeds), AZ6, AZ6 + P and AZ6 + GB for each of the concentrations of NaCl used. The GB or P at 5 mM was added to the different NaCl concentrations. The treated seeds were sown (one seed per pot) at a depth of 1 cm from the surface. The experiment was repeated 6 times and was conducted for 45 days in a growth chamber (phytotron) with an average day/night temperature of 26 and 16°C, respectively and a photoperiod of 16 h light of 2100 lux. Soil moisture was adjusted and constantly maintained during the experiment by watering with sterile distilled water. At stage four to five leaves, the plants were harvested and washed with distilled water. The roots and shoots were collected separately. Their sizes and fresh and dry weights (after 72 h at 65°C) were determined. The dosages of chlorophyll, amino acids, proline and the Na^+ and K^+ contents were also performed.

Determination of chlorophyll

Chlorophylls *a* and *b* were determined according to the method of Arnon (Arnon, 1949). 0.5 g of the shoots of each sample were cut into small segments (0.5 cm) and homogenized in 10 ml of acetone at 80% and stored at -10°C overnight. The organic extract was centrifuged at 14000 rpm/5 min and the absorbance of supernatant was measured at 663 and 645 nm to determine the chlorophyll *a* and *b*, respectively.

CH*a* ($mg.l^{-1}$) = 12.41 OD (663) - 2.59 OD (645).
CH*b* ($mg.l^{-1}$) = 22.9 OD (645) - 4.68 OD (663).
CH*t* = CH*a* +CH*b*
CH*a*: chlorophyll *a* concentration.
CH*b*: chlorophyll *b* concentration.
CHt: total chlorophyll concentration.

Content of the Na^+ and the K^+ in shoots and roots

The concentrations of the Na^+ and the K^+ in the shoots and roots were determined by flame spectrophotometer after digestion of the solids (0.1 g of shoots and 0.05 g of roots) in 10 ml of H_2SO_4 98% and 3 ml of H_2O_2 30% during 5 h according to the method of Skoog et al. (2000).

Determination of soluble amino acids and proline

Samples of shoots and roots were stored below -15°C before analysis. The extraction was conducted using the method described by Naidu (1998): 500 mg of shoots were placed in centrifuge tubes containing 5 ml of methanol: chloroform: water (60:25:15). The sealed tubes were heated in a water bath at 60°C for 2 h and centrifuged at 10000 rpm/10 min. The supernatant was then used for assays of soluble amino acids and proline.

Soluble amino acids

1 ml of acetic acid/sodium acetate buffer (pH 4.3) and 1 ml of ninhydrin (5% in ethanol) were added to 1 ml of supernatant. The samples were stirred and heated in a water bath (95°C) for 15 min. The absorbance was determined at 570 nm.

Proline

Proline was determined by a rapid method developed by Singh et al. (1973): 1 ml of the supernatant, 4 ml of ninhydrin solution, 4 ml of glacial acetic acid and 1 ml of distilled water were introduced into 10 ml centrifuge tubes. This mixture was heated in a water bath (90°C) for 45 min and cooled to room temperature. The absorbance was determined at 520 nm.

Statistical analysis

All the data were the mean of six repetitions for growth parameters and three repetitions for other determinations. Two-way analysis of variance (ANOVA) was conducted with the multifactorial Assistat 7.6 Beta software. When an interaction of two-way ANOVA was smaller than 0.05, the Tukey´s HSD test was realized.

RESULTS

Strain certification

Results showed that this AZ6 strain was identified as *Azotobacter chroococcum*. *A. chroococcum* was rod shape, motility occurred through the use of peritrichous flagella, cysts-forming, positive to oxidase and catalase test with insoluble brown or brown-black pigmentation and darken with age, utilized mannitol, malonate and not rhamnose. Partial 16S rDNA sequences confirmed that the AZ6 strain (accession number KT339176) belonged to *A. chroococcum* species with 99% 16S rDNA similarity (Figure 1).

Morphological parameters

Analysis of variance of fresh and dry weight of roots and shoots, root elongation and plant height in both wheat varieties showed that salinity had a significant negative effect on these growth parameters ($p \leq 0.05$) (Table 1). The treatment effect was significant for both wheat varieties. Inoculation by *Azotobacter chroococcum* AZ6 in the presence or absence of P or GB reduced significantly the salt stress effect on these parameters (Figures 2 to 4). The differences between the two varieties were low but with a fresh and dry weight higher in Waha.

Chlorophyll

Salinity reduced contents of chlorophyll *a*, *b* and total ($p < 0.05$) in both varieties but with a lower effect in Waha (Table 2). Treatment by inoculation in the presence or absence of P or GB improved significantly the chlorophyll *a* and total at all levels of salinity. However, these were higher at 100 and 200 mM for plants inoculated in the presence of P or GB and the effect of these factors was not visible on chlorophyll *b* (Figure 5).

Amino acids

Amino acid content was significantly increased under the salt stress (Table 2, Figure 6). This increase was from 30 to 40% at 100 mM and up 73 to 85% at 200 mM in Waha and Bousselam, respectively. Inoculation by *A.*

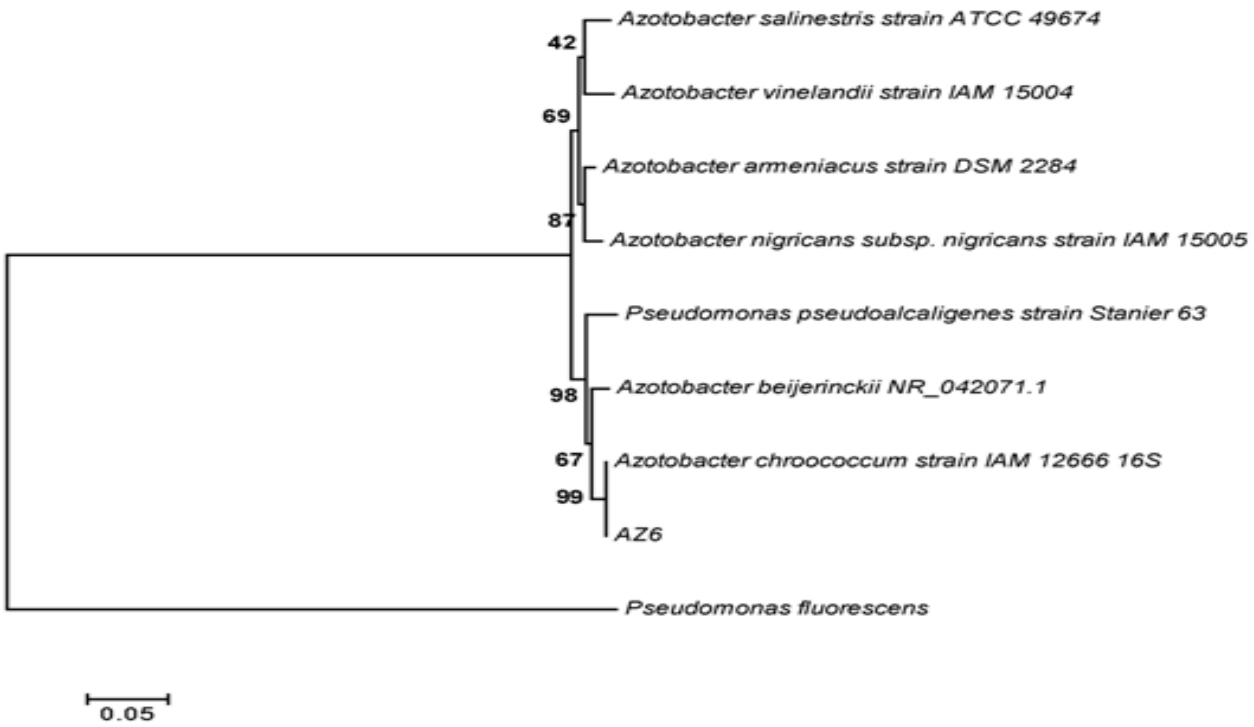

Figure 1. Unrooted phylogenetic tree based on a comparison of the 16S ribosomal DNA sequence of *Azotobacter* (KT339176) and some of their closest phylogenetic relatives (Validly published strains), The tree was created by the neighbor-joining method, The numbers on the tree indicates the percentages of bootstrap sampling derived from 1000 replications, Bar inferred nucleotide substitutions per nucleotides.

Table 1. Analysis of variance summaries (mean squares) of the data for fresh and dry weight, plant height and root length.

Source of variation	df	Shoots fresh Weight (g)	Shoots dry weight (mg)	Roots fresh weight (g)	Roots dry weight (mg)	plant height (cm)	root length (cm)
Treatment (T)	3	14.0200**	83.9662**	18.2932**	14.1300**	1.3319*	14.2141**
Salinity (S)	2	25.8154**	139.1682**	77.7720**	324.0321**	408.100**	329.3861**
S×T	6	2.8527*	4.0371**	3.8491**	31.6417**	8.8541**	1.4071^{ns}
Variety (V)	1	34.5141**	197.4619**	17.3274**	41.1894**	778.94**	26.2284**
S×V	3	2.7577^{ns}	21.7425**	21.2982**	18.2884**	5.5958**	3.5439*
V×T	2	0.0008^{ns}	59.6306**	9.6882**	1.1260^{ns}	15.0568**	3.2453*
S×V×T	6	2.138^{ns}	4.7669**	9.9206**	6.2064 **	1.7873^{ns}	1.8952^{ns}
Error	60	0.253122	21717361	0.02669	2.20650	0.033368	1.923021

*,**:Significative at a level of 5% (p≤0.05) and 1% of probability (p≤0.01), respectively, ns: Non-significative (p≥0.05), df: Degree of freedom.

chroococcum AZ6 reduced these contents. The content of shoot amino acids was lower in the presence of proline or GB at 134 or 167 µg in Waha and 153 or 176 µg in Bousselam.

Proline

Analysis of endogenous proline content in shoot showed that the two varieties of wheat (Waha and Bousselam) strongly accumulated proline under saline conditions. Inoculation by *A. chroococcum* AZ6 decreased the proline content in shoot of the two varieties under the salt stress (Table 2, Figure 7). The exogenous application of P or GB reduced further this content. This effect was visible especially in Waha. In non-saline conditions, the effects of the inoculation treatment and/or application of proline or glycine betaine were not significant.

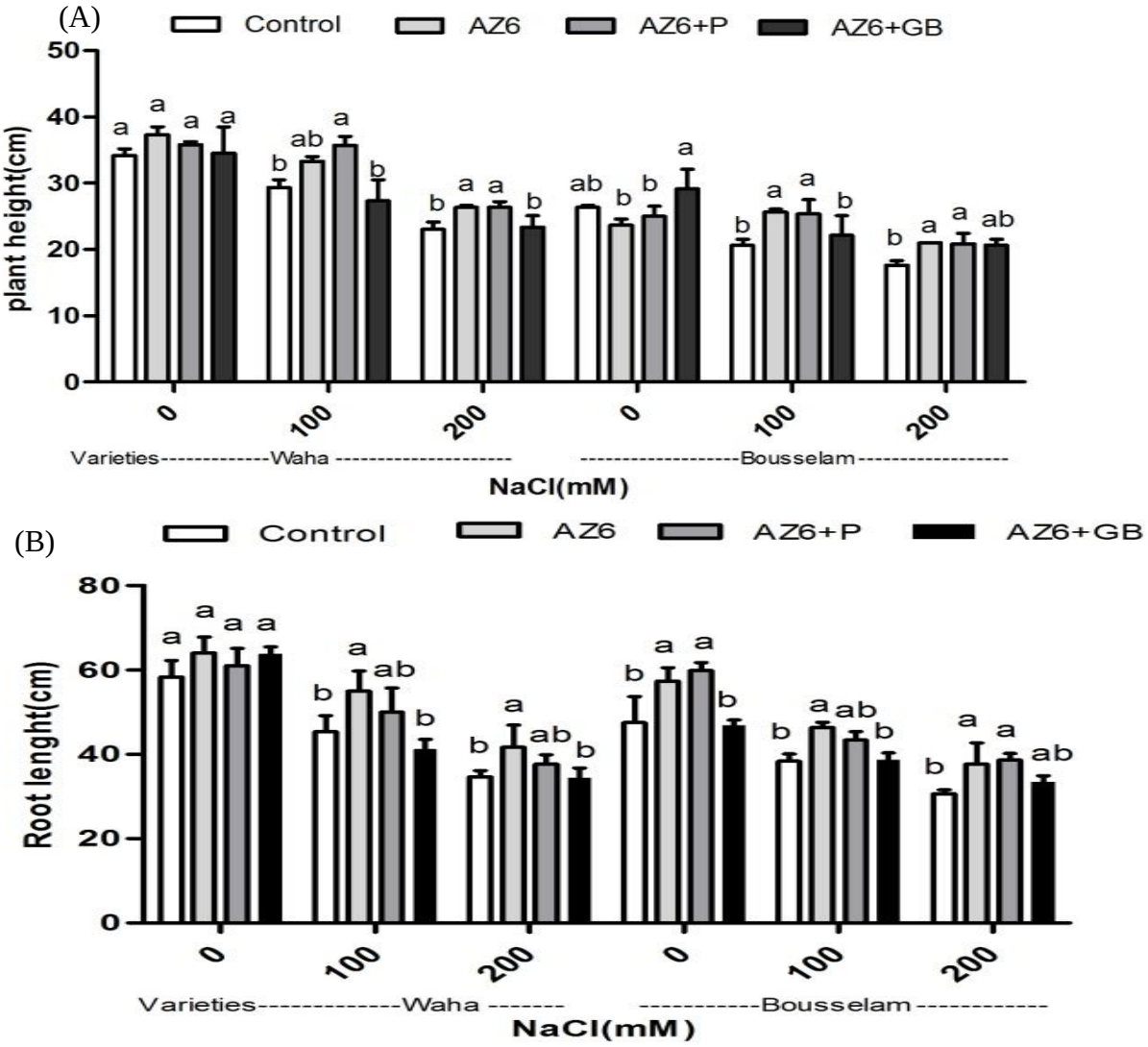

Figure 2. Plant height (A) and root length (B) in two wheat durum varieties at different treatments before and after exposure to 100 and 200 mM NaCl stress, results are shown as Mean±standard error (p≤0.05) from six replicates

The Na^+ and K^+ content in shoots and roots

Salinity also had a significant effect on the Na^+ concentration in shoots and roots in both varieties of wheat ($P \leq 0.01$) (Table 3, Figure 8). This ion increased systematically with the level of salt. However, inoculation with *A. chroococcum* AZ6 in the absence or presence of P or GB reduced the accumulation of Na^+. The accumulation of the Na^+ was more observed in the shoots than in the roots in Bousselam variety. However, the K^+ ions in the roots and the shoots decreased significantly under salt stress in both varieties (Table 3, Figure 9). This decrease was less pronounced for the inoculated samples which were treated with exogenous P or GB. This decrease was also lower in the shoot in Waha for the same samples.

DISCUSSION

The results of experiments on hydroponic medium showed clearly that salt stress led to a reduction in the growth in both wheat varieties resulting in a significant decrease in root length, height of the plant and fresh and dry weight of shoots and roots. These results were similar to those recorded in many plants. In radish (*Raphanus sativus*), for example, the dry weight decreased at high salinity and about 80% of the growth reduction can be attributed to the decrease of the expansion of the leaf area (Marcelis and Hooijdonk, 1999) and therefore, a low light interception. Also, salinity reduced the glycophytes growth by modifying the balance of water and ion in the tissues (Munns, 2002). In leaves, this phenomenon was associated with a decrease in turgor as a result of a

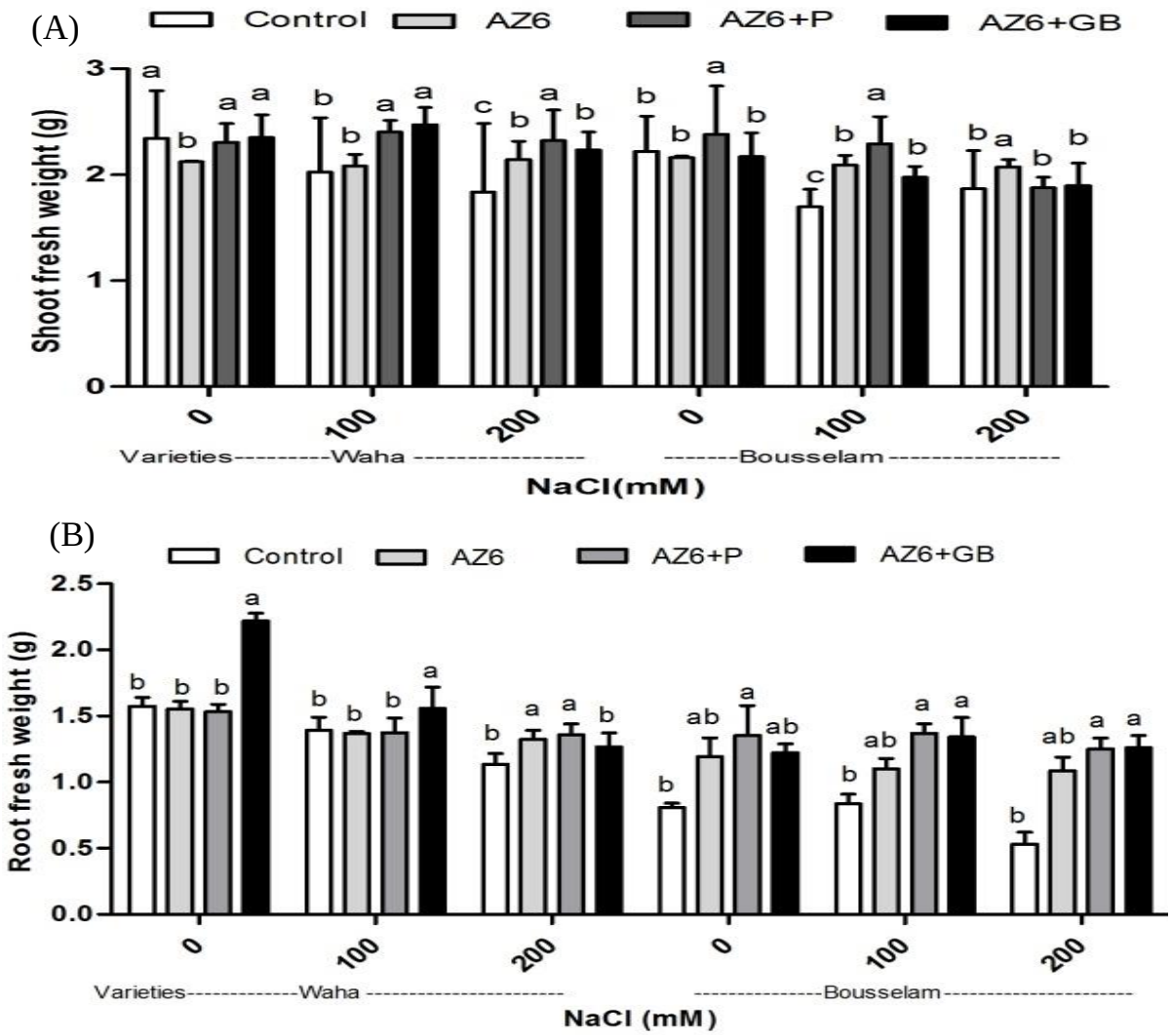

Figure 3. Shoot Fresh (A) and root fresh (B) weight in two wheat durum varieties at different treatments before and after exposure to 100 and 200 mM NaCl stress, results are shown as Mean±standard error (p≤0.05) from six replicates.

decrease in potential gradient of water between the plant and the environment (Levigneron et al., 1995). In addition, the effects of salinity were attributed to other factors including an increase in the rigidity of the cell wall, probably due to a change in its structure or a reduction in its extensibility and a reduction of new cells production rate (Kinraide and Parker, 1990) and/or a toxicity of ions Na^+ and Cl^-, a nutritional deficiency as well as mineral imbalances (Van Volkenburgh and Boyer, 1985). Salt stress also had an effect on the biochemical characteristics. The decrease in chlorophyll concentration under the influence of salinity has been reported by several authors (Del Zoppo et al., 1999). This decrease was attributed to the salt inducing the weakening protein-lipids complexes and an increased activity of chloro-phyllase. Moreover, salinity reduced the biosynthesis of photosynthetic pigments and caused changes in the integrity and composition of the chloroplasts membranes (Günes et al., 1996).

The compartmentation of ions between the organs (roots/aerial parts), tissue (epidermis/mesophyll) or between cellular compartments (vacuole/cytoplasm) was one of the mechanisms of adaptation to salt stress. Generally, in tolerant plants (as opposed to sensitive plants), the Na^+ ion was well distributed in the vacuole (Cheeseman, 1988). The effect of salt stress also increased the absorption of Na^+ through the roots and the leaves in two wheat varieties (Günes et al., 1996). The same observations have been reported in two barley genotypes in which the salt caused the migration of the Na^+ in the aerial parts with greater accumulation through the leaves compared to roots. The mechanisms of the Na^+ transport in the leaves and those of its root absorption appeared to be regulated separately. However, a gain of

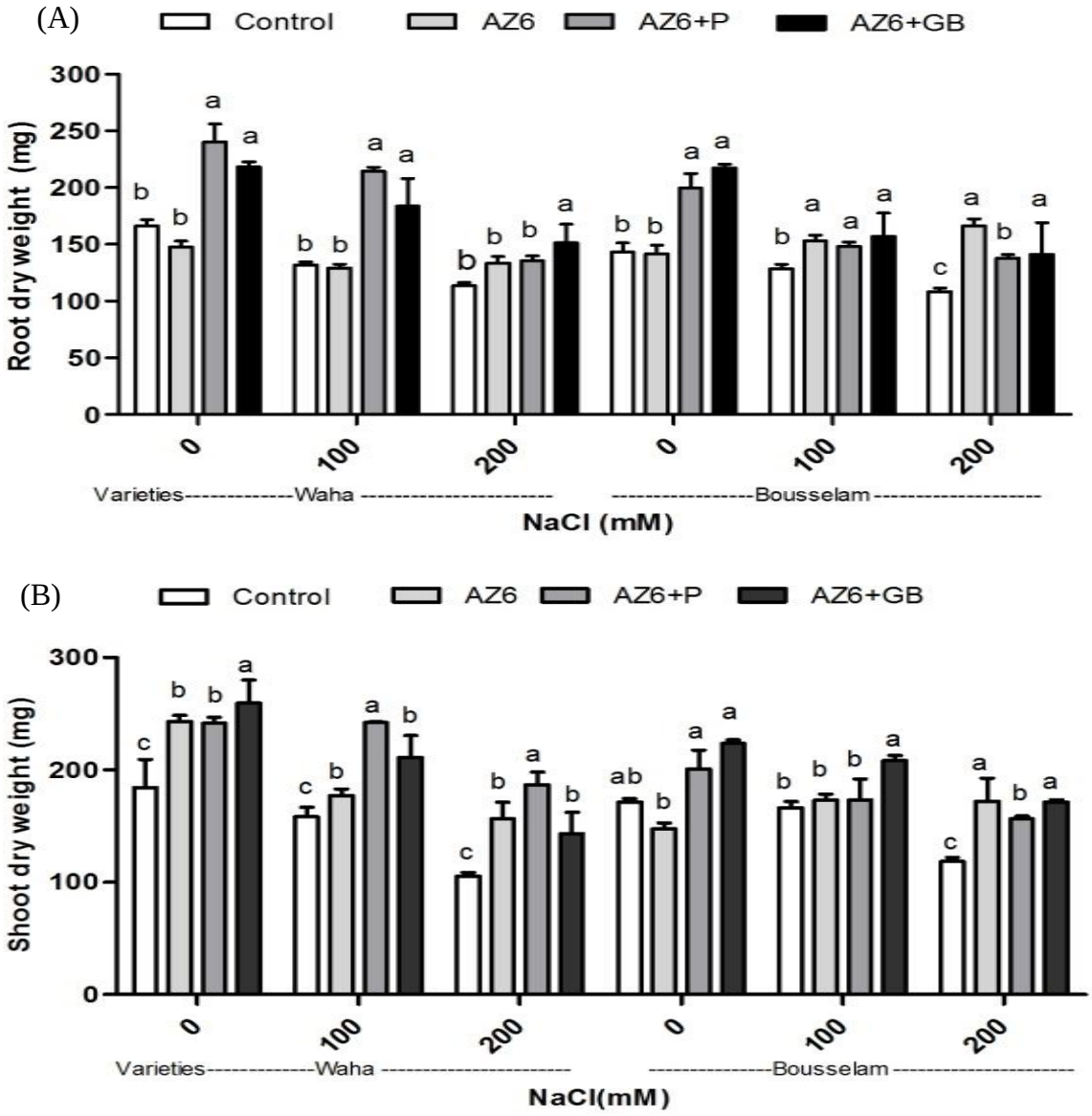

Figure 4. Shoot dry (A) and root dry weight (B) in two wheat durum varieties at different treatments before and after exposure to 100 and 200 mM NaCl stress, results are shown as Mean±standard error (p≤0.05) from six replicates.

Table 2. Analysis of variance of summaries (mean squares) of the data of shoot content chlorophyll *a*, *b* and *a* + *b*, proline and amino acids.

Source of variation	Df	CH*a* (mg/g)	CH*b* (mg/g)	CH*a*+*b* (mg/g)	Proline (µg/g)	Amino acids (µg/g)
Treatment(T)	3	40.5822**	0.8217^{ns}	28.0090**	96.6844**	44.1325**
Salinity (S)	2	415.5958**	7.3617**	221.4564**	2076.5302**	1159.5963**
S×T	6	4.9636**	2.2059^{ns}	1.6871^{ns}	27.6181**	50.3157**
Variety (V)	1	21.6133**	0.4960^{ns}	24.0971**	95.4289**	43.9669**
S×V	3	11.3114**	2.4376^{ns}	5.7110**	11.0311**	0.7574^{ns}
V×T	2	10.0566**	0.6946^{ns}	8.4330**	21.0890**	0.8473^{ns}
S×V×T	6	0.6632^{ns}	0.1517*	0.9138^{ns}	7.5191**	2.2093^{ns}
Error	24	0.40582	0.06554	0.52567	2.31446	4.93056

*,**Significative at a level of 5% (p≤0.05) and 1% of probability (p≤0.01), respectively, ns: Non-significative (p≥0.05), df: Degree of freedom

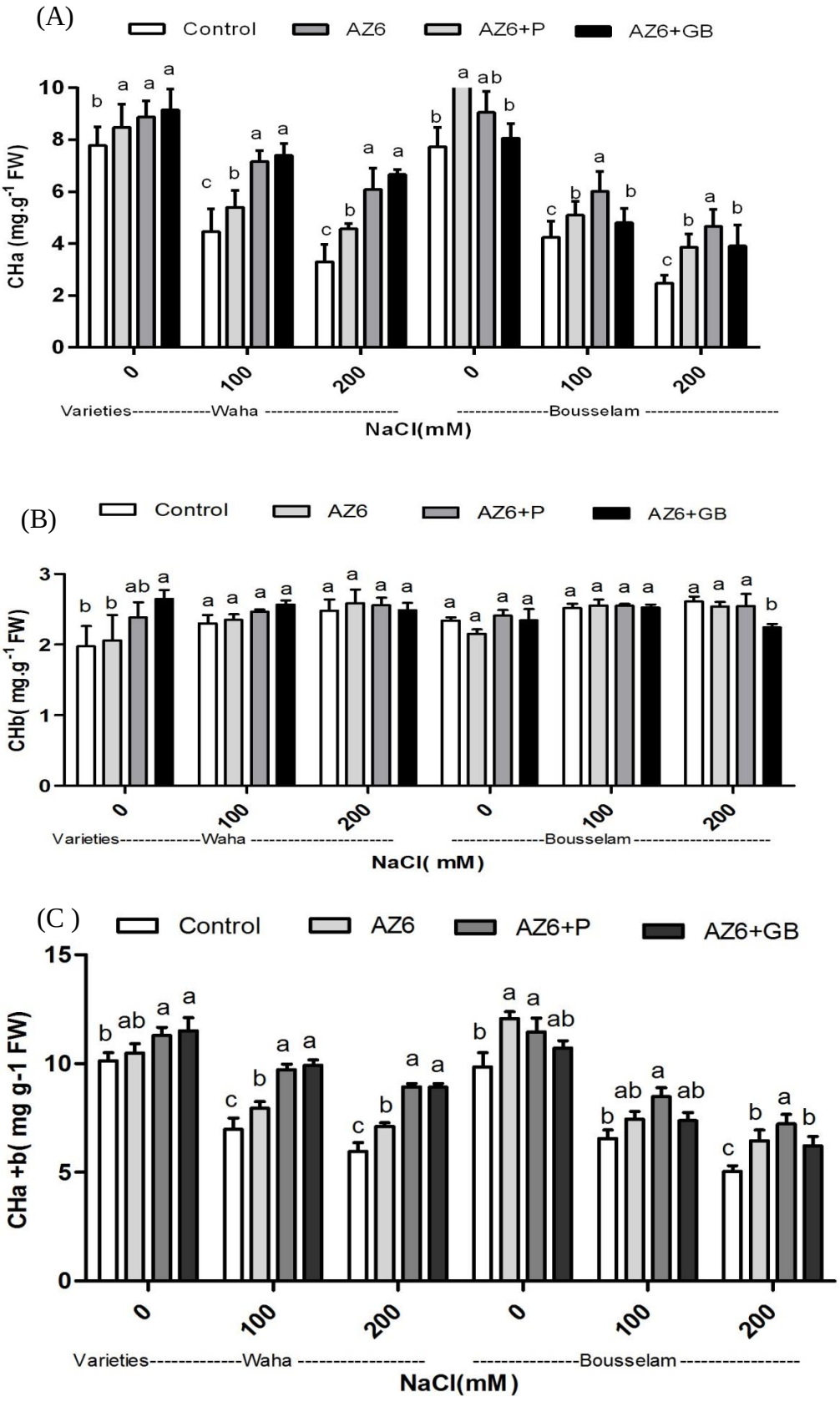

Figure 5. Chlorophyll *a* (A), *b* (B) and *a*+*b* (C) content(mg.g^{-1} FW) in two wheat durum varieties at different treatments before and after exposure to 100 and 200 mM NaCl stress, results are shown as Mean±standard error (p≤0.05) from three replicates.

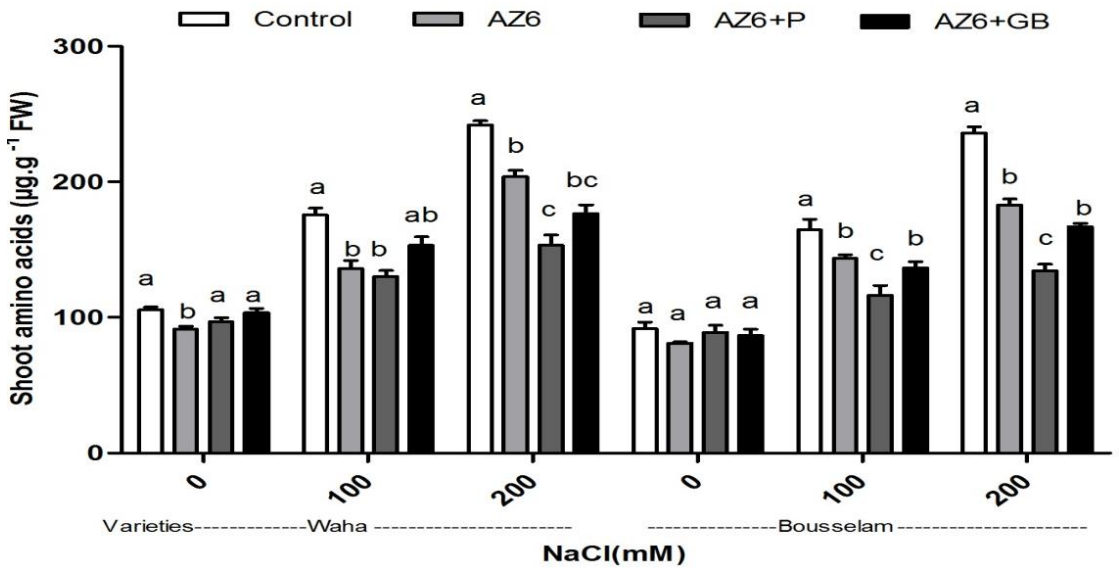

Figure 6. Shoot amino acids content (µg.g^{-1} FW) in two wheat durum varieties at different treatments before and after exposure to 100 and 200 mM NaCl stress, results are shown as Mean±standard error (p≤0.05) from three replicates.

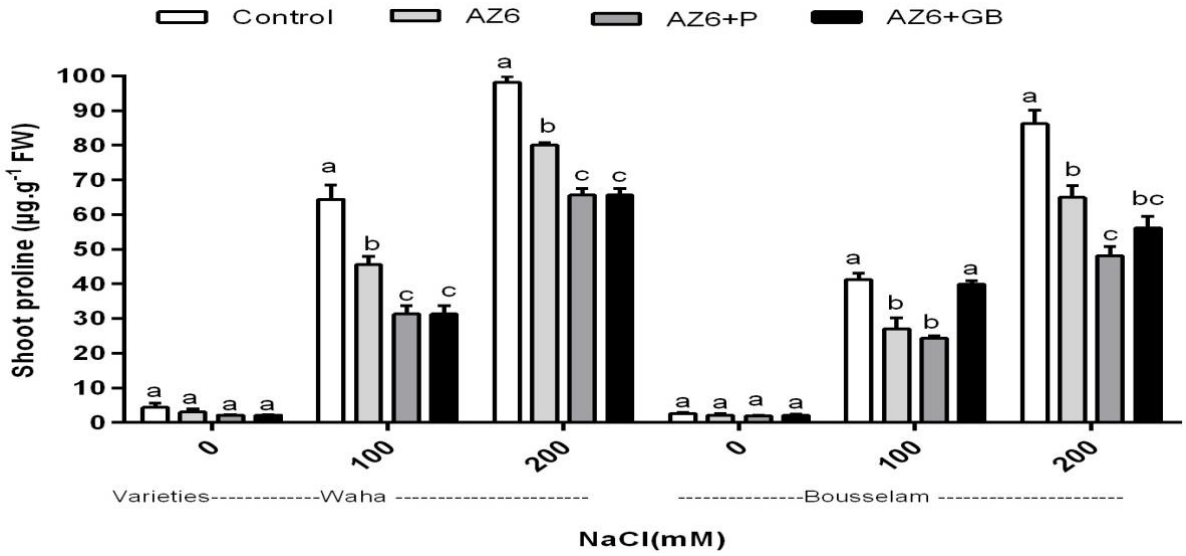

Figure 7. Shoot proline content(µg.g^{-1} FW) in two wheat durum varieties at different treatments before and after exposure to 100 and 200 mM NaCl stress, results are shown as Mean±standard error (p≤0.05) from three replicates.

tolerance was observed in plants with improved ability to recirculate sodium, which protected the aerial parts from the saline invasion (Munns, 2002).

In the same context, the content of the K^+ decreased in the roots and leaves in both varieties of wheat under salinity effect. The same findings were observed in tomato plants grown in the presence of NaCl at 50 and 100 mM (Heuer, 2003). This salt inhibitory effect on the absorption of the K^+ existed also in wheat and in the olive (Ottow et al., 2005). These observations were attributed to a competitive interaction between the Na^+ and the K^+ at the absorption sites of the plant. Reducing the concentration of the K^+ resulted in a reduction of growth by decrease of the osmotic adjustment and the maintenance of the turgor (Bernstein et al., 1995).

The concentrations of amino acids and proline of the shoots increased substantially and reached a remarkable content when the two varieties of wheat were subjected

Table 3. Analysis of variance of summaries (mean squares) of the data for concentrations of Na^+ and K^+ in shoots and roots.

Source of Variation	df	Na^+ shoots	K^+ shoots	Na^+ roots	K^+ Roots
Treatement(T)	3	10.3670*	5.6212*	29.1332**	6.2192*
Salinity(S)	2	17.4079**	280.5441**	320.7020**	1084.6339**
S×T	6	184.9180**	1.8350^{ns}	8.8642**	11.6436**
Variety (V)	1	25.4238**	0.0965^{ns}	58.0627**	5.6434*
S×V	3	0.1888^{ns}	0.0839^{ns}	0.2287^{ns}	3.0335*
V×T	2	3.1246*	6.4001**	14.8168**	3.0335*
S×V×T	6	4.5620**	2.6609*	4.7274^{ns}	2.6055*
Error	24	1.38218	2.76145	0.78264	1.15570

*,**Significative at a level of 5% (p≤0.05) and 1% of probability (p≤0.01), respectively, ns: Non-significative (p≥0.05), df: Degree of freedom.

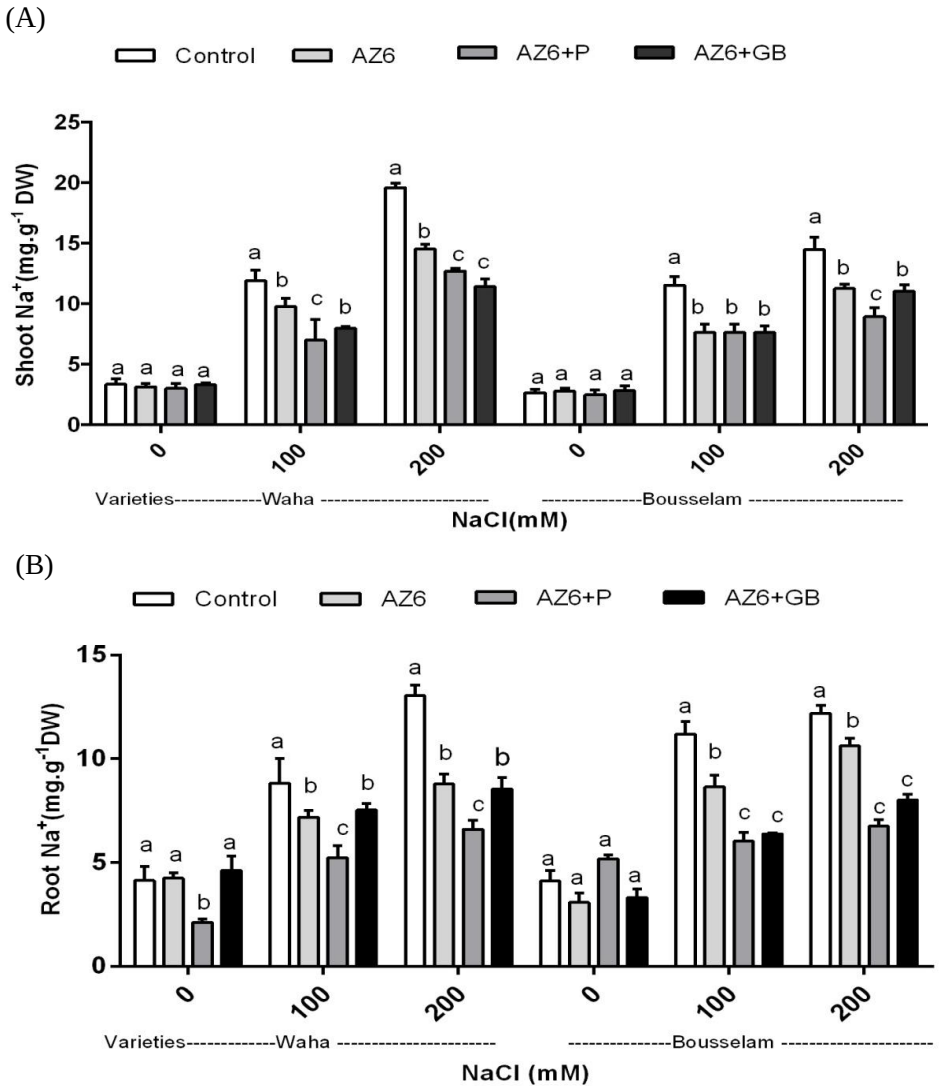

Figure 8. Shoot an root Na^+ content($mg.g^{-1}$ DW) in two wheat durum varieties at different treatments before and after exposure to 100 and 200 mM NaCl stress, results are shown as Mean±standard error (p≤0.05) from three replicates.

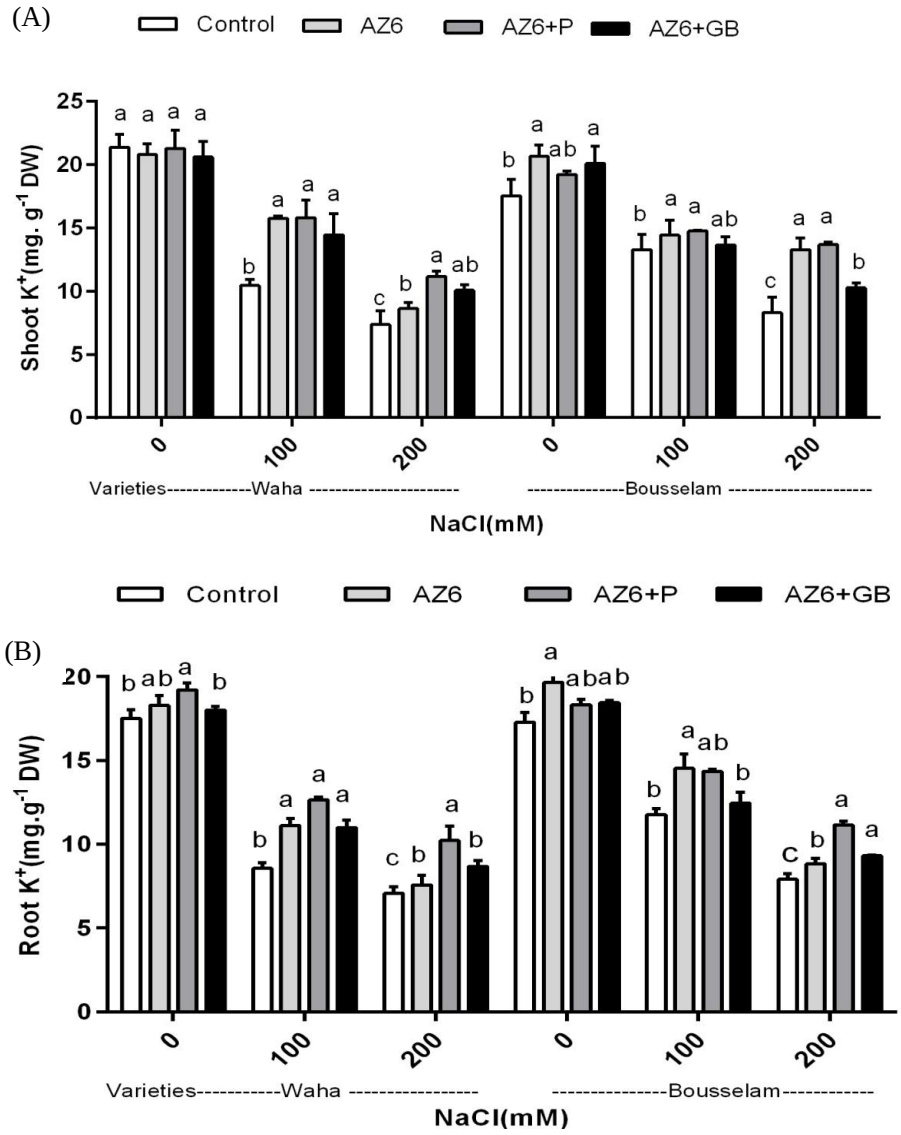

Figure 9. Shoot (A) and root (B) K^+ content($mg.g^{-1}$ DW) in two wheat durum varieties at different treatments before and after exposure to 100 and 200 mM NaCl stress, results are shown as Mean±standard error ($p \leq 0.05$) from three replicates

to salt stress. According to Abd-El-Baki et al. (2000) increased salinity caused an accumulation of amino acids in foliar and root maize. The shoots were more affected by this accumulation. The most important amino acids involved were alanine, arginine, glycine, serine, leucine, valine, proline and non-protein amino acids such as citrulline and ornithine (Mansour, 2000). Proline was probably the most widely accumulated amino acid by plants but also by other organisms (McCue and Hanson, 1990).

In many plant species (including cereals), the presence of proline in high concentrations was one of the manifestations of water and salt stress (Ali Dib et al., 1992). On one hand, the correlation between proline content and concentration of Na^+ ion confirmed the remarkable osmoregulatory role of proline (Chowdhury et al., 1993).

On the other hand, it was also reported that sensitive wheat varieties significantly accumulated proline concentrations higher than tolerant varieties (Lutts et al., 1999). Proline played several physiological roles in plants subjected to salt stress (osmoregulation, a source of energy, of carbon and nitrogen and a senescence signal) (Aspinall and Palleg, 1981). Its accumulation in the plant tissues under stress conditions was reported as the result of a decrease in its degradation due to an inhibition of proline dehydrogenase, an increase of its biosynthesis by expression of a gene encoding the pyrolline-5-carboxylate

synthetase (Lutts et al., 1999), and a decrease in the synthesis or protein hydrolysis (Viégas and Silveira, 1999). However, the significance of the accumulation of proline in osmotic adjustment is still unclear and varies by species (Rhodes and Hanson, 1993).

The salt inhibitory effect was due to differential genetic potential. In this study, the salinity effect on the morpho-biochemical parameters of both wheat varieties revealed a tolerance of Waha more important than Bousselam under different treatments. This tolerance was explained by the acquisition of resistance genes or a natural adaptation to salt stress which is related to the ability of the plant to restrict the accumulation of the Na^+ ion while promoting that of the K^+ ion and those of osmolytes, such as proline (Botella et al., 2005).

Under salt stress, the addition of glycine betaine (5 mM) or proline (5 mM) stimulated root length, the morphological parameters of the growth, the chlorophyll *a* and total and the K^+ content of roots and shoots. However, the amino acids, the endogenous proline and the Na^+ ions concentrations were reduced. The salt stress induced a higher level of proline content, whereas GB and proline application reduced the proline accumulation when compared with salt treatment without GB and proline, suggesting that proline accumulation is just a symptom of salt stress rather than a cause of tolerance (Ashraf, 1989). These results are in agreement with those of Shaddad (1990) and Gagnon and Dansereau (1990) on *Raphanus sativus* (turnip), *Gossypium hirsutum* and *Vicia faba* (broad beans), respectively. These observations were explained by the fact that glycine betaine, zwitterionic ampholyte can interact directly with the ions accumulated to protect membranes from the deleterious effects of toxic ions (Papageorgiou and Murata, 1995). The addition of proline or glycine betaine promoted a smaller ion transport due to a decreased respiration. These osmolytes also increased cell turgor through osmotic adjustment and contributed to the increase in stomatal conductance in leaves (Heuer, 2003).

Inoculation of durum wheat seeds by *A. chroococcum* AZ6 significantly increased the growth parameters plant height, root length and fresh and dry weight of root and shoot under salt stress. The chlorophyll and the potassium contents were improved while the concentrations of amino acids, proline and sodium were reduced. These results were reported by many authors (Nadeem et al., 2006; Chaudhary et al., 2013). According to Glick et al. (1998), the use of PGPR (for example, *Pseudomonas*) as inoculant of wheat seeds in saline soils improved the plant height, root length, the grain yield, chlorophyll content and the ratio K^+/Na^+. The role of *Azotobacter* in the production of growth-promoting substances and plant improving resistance to abiotic and biotic stresses must also be considered (Chaudhary et al., 2013; Sahoo et al., 2014).

Moreover, it was established that the PGPR strains producing exopolysaccharides (EPS) that bind with the Na^+ ions were capable of reducing the concentration and absorption of this ion in root and thus attenuated the salt stress in plants (Ashraf et al., 2004).

According to many authors, the addition of osmolytes and essentially proline can have a direct and beneficial effect on both the survival and growth of *A. chroococcum* AZ6 and tolerance of the plant to salt stress. Indeed, the middle salinity was a stressful environment for the rhizobacteria. The exposure of the bacteria to conditions of high osmolarity decreased the activity of water in their cytoplasm (Epstein, 1986) and led to harmful effects on cell proteins and other macromolecules. In addition, the salinity reduced the number of root colonizing bacteria. The contribution of osmolytes (proline or glycine betaine) in the environment played an important role in the adaptation of these rhizobacteria to salt stress and as osmoprotectant or as carbon and nitrogen sources (Alloing et al., 2006).

The addition of osmolytes, glycine betaine and essentially proline, attenuated the salinity effects by improving the morpho-biochemical parameters. The use of *A. chroococcum* AZ6 as an inoculant and providing osmoprotective molecules will be, therefore, promising biofertizant in arid and saline soils.

Conflict of interests

The authors have not declared any conflict of interest.

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In vitro evaluation of probiotic potential of five lactic acid bacteria and their antimicrobial activity against some enteric and food-borne pathogens

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Lactic acid bacteria (LAB) present many important properties in food manufacturing, such as improvement of physical characteristics and the production of lactic acid that aids in the increase of the shelf life of food products. Also, LAB can suppress growth of pathogens, control of serum cholesterol level, modulate immune system, and improve lactose digestion. Five standard (*Lactobacillus paracasei, Lactobacillus helveticus*, *Lactobacillus fermentum, Bifidobacterium longum* and *Lactococcus lactis*) lactic acid bacterial strains were screened for probiotic potential properties and their ability to antagonize the growth of some enteric pathogens isolated from patients suffering from acute gastroenteritis. The five strains of LAB were resistant to acidic pH and bile salts. *Lactobacillus* strains showed protein and starch digesting capability on agar plate while *B. longum* ATCC 15707 and *L. lactis* subsp. *lactis* ATCC 11454 showed only protein digestion. In addition, *Lactobacillus* strains showed antagonistic effects against all pathogenic strains tested. *L. paracasei* and *L. helveticus* [culture and cell-free culture supernatant (CFCS)] exhibited the highest antagonistic activity against the tested pathogens followed by *L. fermentum.* While *B. longum* and *L. lactis* subsp. *lactis* showed weak or no activity against the tested strains. *L. paracasei*, *L. helveticus*, and *L. fermentum* showed potential to be used as probiotic strains with considerable good antagonistic activity against the most important enteric pathogens.

Key words: *Lactobacillus paracasei*, *Lactobacillus helveticus*, *Lactobacillus fermentum*, *Bifidobacterium longum* subsp. *longum*, *Lactococcus lactis* subsp. *lactis*, potential probiotic, antagonistic activity.

INTRODUCTION

Foodborne bacterial pathogens, such as *Escherichia coli*, *Salmonella*, *Shigella*, *Yersinia enterocolitica* and *Staphylococcus aureus* can cause diseases that ranged from mild diarrhea to severe illness with high mortality (Petri Jr. et al., 2008). Worldwide, diarrheal disease remains one of the most important causes of morbidity and mortality among infants and children (Ryan, 2004) with 15 billion episodes and 15 to 25 million deaths estimated to occur annually among children aged <5 years (Kosek et al., 2003; Black et al., 2003; Parashar et al., 2003). Although, the use of antimicrobials can limit the growth of enteric pathogens and the use of glucose-

electrolyte oral rehydration therapy (ORT) has dramatically reduced acute mortality from dehydration caused by diarrhea. Antimicrobial resistance becomes a common finding increasing over time and the rates of morbidity remain as high as ever (Kosek et al., 2003, Andersson and Hughes, 2010).

According to the working group FAO/WHO (2002), probiotics are defined as live microorganisms which when administered in adequate amounts confer a health benefit on the host. Probiotics can reduce the duration of diarrhea by 0.7 days as well as the frequency of diarrheal episodes already in the first hours (Van Niel et al., 2002) and their consumption is, therefore, recommended in case of acute gastroenteritis starting from the onset of symptoms (Aureli et al., 2011). Probiotics help keep up the balance between harmful and beneficial bacteria in the gut, thus maintaining a healthy digestive system (Ouwehand et al., 2002). *Bifidobacterium* and *Lactobacillus* are naturally inhabitants of the human intestinal microbiota, some strains have a satisfactory tolerance to the gastrointestinal transit, good survival in food or pharmaceutical supplements and health-promoting effects; so, the strategy of probiotic supplementation is the reinforcement of the intestinal microbiota, at least transitory, with health-promoting bacteria to benefit the intestinal balance (Farnworth, 2008).

The effect of probiotics ranges from regulation of bowel activity and well-being to more specific actions, such as, antagonistic effect on the gastroenteric pathogens like *Clostridium difficile*, *Campylobacter jejuni*, *Helicobacter pylori*, rotavirus, etc. (Doron and Gorbach, 2006).

There are various proposed mechanisms that describe how different probiotics work and they vary depending on the strain of probiotic used. The effects of probiotics also depend on the dosage and route of administration (Upadhyay and Moudgal, 2012). Some of these mechanisms are the production of bacteriocins, such as nisin [approved by the US Food and Drug Administration (FDA) since last decade for food preservation and shelf life extension] (Yateem et al., 2008; Collins et al., 2012) or lowering the pH by producing acidic compounds like lactic acid (Psomas et al., 2001). Competition with other infectious bacteria for nutrients and receptors mediating colonization to host cells (Piard and Desmazeaud, 1991). A few strains are also known to produce active enzymes which inhibit other pathogenic bacteria (Gotcheva et al., 2002). The most common application of probiotics is for dairy production such as yogurt, cheese, and ice cream. Recently, several studies were done for the use of probiotic bacteria in preventing antibiotic-associated diarrhea and *C. difficile* infections (McFarland, 2009). In addition, many species have been suggested to be effective in alleviating gastrointestinal pathogenic bacterial infections both *in vitro* and *in vivo* (Chung and Yousef, 2010; Forestier et al., 2001; Wong et al., 2013), without any pathogenic effect on human and animals.

Based on previously reported beneficial properties of probiotics, our study aimed to evaluate the probiotic properties of *Lactobacillus paracasei* ATCC 25598, *Lactobacillus helveticus* ATCC 15009, *Lactobacillus fermentum* EMCC 1346, *Bifidobacterium longum* subsp. *longum* ATCC 15707 and *Lactococcus lactis* subsp. *lactis* ATCC 11454, including their antagonistic activity against some enteric and food borne-pathogens.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Five lactic acid bacterial strains (LAB) were supplied from Microbiological Resources Center (Cairo MIRCEN), Faculty of Agriculture, Ain Shams University; these strains were as follows: *L. paracasei* ATCC 25598, *L. helveticus* ATCC 15009, *L. fermentum* EMCC 1346, *B. longum* subsp. *longum* ATCC 15707, and *L. lactis* subsp. *lactis* ATCC 11454. *Lactobacillus* strains were cultured in De Man, Rogosa and Sharpe (MRS) broth with tween 80 (Biolife Italiana, Milano, Italia) anaerobically at 37°C, *B. longum* subsp. *longum* ATCC 15707 were cultured in tryptone soy (TS) broth (Lab M Limited, United Kingdom) anaerobically at 37°C, however, *L. lactis* subsp. *lactis* ATCC 11454 were cultured in Brain heart infusion (BHI) broth (Lab M Limited, United Kingdom) aerobically at 37°C. Also, standard pathogenic bacterial strains were supplied from the same previous center. These strains were *E. coli* ATCC 8739 and *S. aureus* ATCC 6538 and were cultured in Nutrient broth (Lab M Limited, United Kingdom) aerobically at 37°C.

Gram-negative enteric bacteria were isolated from patients suffering from acute gastroenteritis attending Minia University Hospital, Minia, Egypt. These isolates were identified according to standard laboratory procedures (Collee et al., 1996; MacFaddin, 2000). Five isolates of *E. coli* (E1, E2, E3, E4 and E5), one isolate of *Y. enterocolitica* (Y1), three isolates of *Citrobacter koseri* (C1, C2, C3) and one isolate of *Shigella sonnei* (Sh1) were used in this work.

Starch, protein and lipid digesting capabilities

The protein, lipid and starch digesting capabilities of *Lactobacillus* strains were evaluated using modified MRS agar containing 2.8% skimmed milk (HiMedia Laboratories Pvt. Ltd, India), 1% tributyrin (Sigma-Aldrich, St Louis, USA), and 0.2% soluble starch (Oxford lab, India), respectively. The overnight cultures of *Lactobacillus* strains (10 µl) were dropped on the modified MRS agar and incubated at 41°C for 24 h. The diameters of the halo zone on the agar plate were then measured. The digesting capability of the tested strains was classified as positive when the diameters of the

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clear zone were more than 1 mm. Each assay was performed in triplicate. The same test was performed for *B. longum* subsp. *longum* and *L. lactis* subsp. *lactis* using TS agar and BHI agar, respectively instead of MRS agar (Thongsom, 2004).

Sensitivity to bile salts and acidic pH

Resistance to bile salts was evaluated by the ability of *Lactobacillus* strains, *B. longum* subsp. *longum* and *L. lactis* subsp. *lactis* to grow on MRS agar, TS agar, and BHI agar, respectively, containing 2% (w/v) bile salt. The cultures were checked after 48 h incubation at 37°C. Resistance to acidic pH (pH 3.0) was analyzed by centrifuging the overnight cultures of the *Lactobacillus* strains at 6000 ×g for 15 min at 4°C and re-suspending the pellets in the same volume of 0.9% (w/v) NaCl, pH 3.0. The suspensions were then incubated at 37°C for 3 h. After incubation and culture centrifugation, the resulting pellets were plated onto MRS agar and incubated at 37°C for 48 h. Resistance to acidic pH (pH 3.0) was performed for *B. longum* subsp. *longum* and *L. lactis* subsp. *lactis* using TS agar and BHI agar, respectively instead of MRS agar (Nouri et al., 2010).

Assay of LAB culture effect on the growth of the tested enteric pathogens

Antibacterial activity of LAB was studied using the agar diffusion method (Makras and Vuyst, 2006). The indicator strains used in this study were Gram-negative enteric bacteria isolated from patients suffering from gastroenteritis. In addition, some standard bacterial species potentially pathogenic to humans, such as *S. aureus* and *E. coli* were used. Indicator strains were cultivated in nutrient broth at 41°C for 18 h. To evaluate the antibacterial activity, *Lactobacillus* strains were cultivated in MRS broth at 41°C for 18 h. However, TS broth and BHI broth were used for cultivation of *B. longum* subsp. *longum* and *L. lactis* subsp. *lactis* respectively. The culture containing 10 µl of LAB (10^8 CFU/mL) was dropped on MRS agar for *Lactobacillus* strains, TS agar for *B. longum* subsp. *longum* and BHI agar for *L. lactis* subsp. *lactis* and incubated at 41°C for 18 h. The LAB on the agar plate were overlaid with 9 ml of soft nutrient agar with 1 ml of culture of indicator strains cultivated overnight (10^6 CFU/ml). The agar plates were incubated at 41°C for 18 h and diameters of inhibition zones on the agar plate were measured. Each assay was performed in triplicate. The antibacterial activity was calculated as follows: antibacterial activity in mm = diameter of inhibition zone - diameter of LAB colony (Musikasang et al., 2009).

Assay of cell-free culture supernatant (CFCS) of LAB on growth of the tested enteric pathogens

Preparation of cell-free culture supernatants: Briefly, *Lactobacillus* strains were grown for 18 h at 37°C in 10 ml of MRS broth; however, TS broth and BHI broth were used for cultivation of *B. longum* subsp. *longum* and *L. lactis* subsp. *lactis*, respectively. The culture containing 10^8 CFU/ml was then centrifuged at 4000 ×g for 10 min. Then the supernatants were collected and sterilized by filteration using CHM© CA syringe filter of 0.20 µm pore size and the cell-free culture supernatants (CFCS) were stored in cryotubes at -20°C (Yu et al., 2013; Pehrson et al., 2015).

Effect of CFCS on the growth of the tested pathogens: The antibacterial activity of LAB strains was investigated against indicator strains by a modified method of Wang et al. (2014) using cell-free culture supernatant (CFCS). Dried and sterilized filter paper discs (6 mm diameter) were then filled with known volume (10 µl per disc) of the CFCS using micropipette. Disc containing the test material were placed on the same agar plate overlaid with 9 ml of soft nutrient agar inoculated with 1 ml of culture of indicator strains cultivated overnight (10^6 CFU/ml) mentioned earlier. After overnight incubation, the diameter of the inhibition zone around disc was measured. Sterilized MRS broth in case of *Lactobacillus* strains, BHI broth in case of *L. lactis* subsp. *lactis* and TS broth in case of *B. longum* subsp. *longum* , all having pH 7.0, were used as control to verify the presence of a possible inhibitory compound in the medium (for example sodium acetate, citrate) (Pehrson et al., 2015). Each assay was performed in triplicate.

RESULTS AND DISCUSSION

Starch, protein and lipid digesting capabilities

Of the tested LAB strains it was found that the three *Lactobacillus* strains exhibited both protein and starch digesting capability, but the lipid digestion was not encountered here. Some strains of LAB have the capability to digest protein, but they cannot digest starch or lipids (Musikasang et al., 2009). Other LAB could utilize protein, lipids and starch (Duangchitchareon, 2006; Kawai et al., 1999; Thongsom, 2004). However, *B. longum* subsp. *longum* and *L. lactis* subsp. *lactis* showed only protein digestion, LAB have developed a complex system of proteinases and peptidases which enable them to utilize casein protein found in milk (Smid et al., 1991). Klaver et al. (1993) reported that *Bifidobacterium* strains were not as proteolytic as other LAB. This may explain why *Bifidobacterium* species grows slowly in milk and may require supplementation of peptides and amino acids from external sources (Dave and shah, 1998). LAB are fastidious microorganisms that require an exogenous source of amino acids or peptides, which are provided by the proteolysis of casein, the most abundant protein in milk and the main source of amino acids (Savijoki et al., 2006). In general, the exploitation of casein by LAB is initiated by a cell-envelope proteinase (CEP) that degrades the protein into oligopeptides that are subsequently taken up by the cells via specific peptide transport systems for further degradation into shorter peptides and amino acids by a concerted action of various intracellular peptidases (Kunji et al., 1996; Christensen et al., 1999). Although most LAB are unable to degrade starch because of the lack of the amylolytic activity, a few exhibit this activity and are qualified as amylolytic lactic acid bacteria (ALAB) which are able to decompose starchy material through the amylases production during the fermentation processes (Asoodeh et al., 2010). Most amylolytic LAB isolated belong to the *Lactobacillus* genus, while few studies reported the existence of amylolytic activity in some strains of *Bifidobacterium* isolated from the human large intestinal tract (Ji et al., 1992; Lee et al., 1997). Amylolytic LAB are mainly used in food fermentation, they are involved in cereal based fermented foods such as European sour rye

Table 1. Resistance to bile salts and acidic pH.

LAB strains	Resistance	
	Bile salts	Acidic pH
Lactobacillus paracasei	+	+
Lactobacillus helveticus	+	+
Lactobacillus fermentum	+	+
Bifidobacterium longum subsp. *longum*	+	+
Lactococcus lactis subsp. *lactis*	+	+

Resistance to bile salts and acidic pH (3) are shown by +.

bread, Asian salt bread, sour porridges, dumplings and non-alcoholic beverage production (Fossi and Tavea, 2013). Few of them are used for the production of lactic acid in single step fermentation of starch (Reddy et al., 2008).

Sensitivity to bile salts and acidic pH

In order to classify LAB as probiotic bacteria, they should be resistant to low pH environment of the stomach and bile salts of the intestinal tract to survive and grow in the GIT to exert their probiotic function effectively (Musikasang et al., 2009).

It was found that the three *Lactobacillus* strains used in the study were able to grow on the MRS agar containing 2% bile salts, and also resist the acidic pH (pH=3), similarly, *B. longum* subsp. *longum* and *L. lactis* subsp. *lactis* showed acceptable resistance to the effect of bile salts and acidic pH and remained viable as shown in Table 1. Resistance to bile salts is important for LAB to become able to colonize and be metabolically active in the small intestine of the host (Strompfová and Laukova, 2007). Bile resistance may be due to expression of bile-resistance related proteins in the bacterial cells (Hamon et al., 2011). In a previous work, *L. paracasei* was included in human pilot studies, as part of a multicentre European project named PROB-DEMO, to assess the ability of probiotics to survive intestinal transit and to examine their influence on the native microbiota of consumers. It was found that it has a proven ability to survive gastric transit and to persist in the colonic environment of humans (Crittenden et al., 2002).

Lankaputhra and Shah (1995) have reported that among nine strains of *Bifidobacterium* spp., *B. longum*, *B. pseudolongum* and *B. infantis* showed the best tolerance to bile salt (1 to 15%). Also, it was reported by Clark et al. (1993) that *B. longum* shows better survival in acidic conditions compared with other *Bifidobacterium* spp. (*Bifidobacterium infantis, Bifidobacterium adolescentis,* and *Bifidobacterium bifidum).* *L. casei* NCDC 63, *L. casei* VT, and *L. casei* C1 could survive after being treated with 2% (ox bile) for 2 h of incubation (Mishra and Prasad, 2005). *Lactobacillus* spp. exhibited survival to bile salt and in the presence of 0.3 mg/L pancreatin (Maragkoudakis et al., 2006); *Lactobacillus salivarius* and *L. crispatus* isolated from chicken gastrointestinal tract showed resistance to acidic pH and bile (Nouri et al., 2010). Some *L. plantarum* strains, e.g. strains LC56 (Hamon et al., 2011) and LP3 (Karasu et al., 2010), were sensitive to bile, *L. casei* strains 2123 and F19 survived rather poorly at pH 2.0 (Charteris et al., 1998), Other *Lactobacillus* strains survived at pH values of 2.5 to 4.5, but lost viability at lower pH values (Conway et al., 1987; Jacobsen et al., 1999; Maragkoudakis et al., 2006).

Bile salts at high concentrations can rapidly dissolve membrane lipids and cause dissociation of integral membrane proteins resulting in the leakage of cell contents and cell death (Begley et al., 2005). It has been suggested that the major effect of bile acids would be the disaggregation of the lipid bilayer structure of the cell membrane. Conjugated bile acids are less inhibitory than free bile acids (cholic and deoxycholic acid, DCA) toward intestinal aerobic and anaerobic bacteria Taurine-conjugated deoxycholic acid (TDCA) was less toxic than DCA. The tolerance to bile salts was initially associated with the presence of bile salt hydrolase activity (Moser and Savage, 2001; Taranto et al., 2006).

Effect of culture suspension and cell-free culture supernatant (CFCS) of LAB on the growth of the tested enteric pathogens

The antibacterial activity of LAB may often be due to the production of organic acids, with a consequent reduction in pH, or to the production of hydrogen peroxide (González et al., 2007). LAB could produce various compounds such as organic acids, diacetyl, hydrogen peroxide, and bacteriocin during lactic fermentations. Levels and types of organic acids produced during the fermentation process depended on LAB species or strains, culture compositions and growth conditions (Lindgren and Dobrogosz, 1990). Lactic acid is the major organic acid in LAB fermentation where it is in equilibrium with its un-dissociated and dissociated forms, and the extent of the dissociation depends on pH.

The antimicrobial effect of organic acids lies in the reduction of pH, as well as the un-dissociated form of the molecules. It has been proposed that the low external pH causes acidification of the cell cytoplasm, while the un-dissociated acid, being lipophilic, can diffuse passively across the membrane. The un-dissociated acid acts by collapsing the electrochemical proton gradient, or by altering the cell membrane permeability, which results in disruption of substrate transport systems (Ammor et al.,

Table 2. Effect of culture suspension and cell-free culture supernatant (CFCS) of LAB on the growth of *E. coli*.

LAB	Pathogens											
	***E. coli* ATCC 8739**		**E1 (38)**		**E2 (41)**		**E3 (11)**		**E4 (34)**		**E5 (16)**	
	LAB culture*	**CFCS****	**LAB culture***	**CFCS****	**LAB culture***	**CFCS****	**LAB culture***	**CFCS****	**LAB culture***	**CFCS***	**LAB culture***	**CFCS****
L. fermentum	22±1.0	++	19±1.2	++	28±0.65	++	32±1.3	+++	29±1.5	++	16±0.4	++
L. helveticus	27±0.6	+	28±0.61	++	36±0.23	++++	45±0.4	++	38±0.25	+++	18±0.33	++
L. paracasei	36±0.45	+++	36±1.21	++++	22±0.33	++	13±1.2	++	6±1.3	++	21±0.7	++++
B. longum subsp. *longum*	1±0.7	++	13±1.5	NE	3±1.0	++	-	NE	13±0.22	++	6±0.18	++
L. lactis subsp. *lactis*	-	++	3±0.41	++	-	NE	2±0.3	NE	5±1.1	++	-	NE

*Inhibition zones ± standard deviation (SD) in mm. **Culture supernatant was used for investigating antimicrobial activity. Diameters of inhibition zone are the mean of three replicates+ diameter of inhibition zone < 2 mm, ++ diameter of inhibition zone between 2 and 7 mm, +++ diameter of inhibition zone between 8 and 12 mm, ++++ diameter of inhibition zone between 13 and 17 mm NE no effect detected.

2006). In general, organic acids have a strong inhibitory activity against Gram negative bacteria (Makras and Vuyst, 2006). Hydrogen peroxide is produced by LAB in the presence of oxygen as a result of the action of flavoprotein oxidases or nicotinamide adenine dinucleotide (NADH) peroxidase. The antimicrobial effect of hydrogen peroxide may result from the oxidation of sulfhydryl groups causing denaturing of a number of enzymes, and from the peroxidation of membrane lipids, thus increasing membrane permeability (Condon, 1987; Kong and Davison, 1980). LAB strains were reported to inhibit the growth of pathogenic bacteria in many studies (Ammor et al., 2006; Bernbom et al., 2006; Collado et al., 2005; Olkowski et al., 2008; Santos et al., 2003). It may also be due to the production of bacteriocins or bacteriocin-like compounds (Gonza´lez et al., 2007). The bacteriocins from the generally recognized as safe (GRAS) LAB have generated a great deal of attention as a novel approach to control pathogens in food-stuffs (Savadogo et al., 2004).

The results of antagonistic effects of the *Lactobacillus* strains, *B. longum* subsp. *longum* and *L. lactis* subsp. *lactis* against 5 types of pathogenic strains are shown in Tables 2, 3, 4 and 5. The three *Lactobacillus* strains showed antagonistic effects against all pathogenic strains tested, but the degrees of antagonism varied among the *Lactobacillus* strains. *L. paracasei* and *L. helveticus* (culture and CFCS) exhibited strong inhibition on the growth of *E. coli* ATCC 8739 (Figure1a and 1b), and the other tested strains followed by *L. fermentum* (Figure 1c), while *B. longum* subsp. *longum* and *L. lactis* subsp. *lactis* showed weak or no activity against the tested strains.

L. paracasei showed the highest activity against *S. aureus* ATCC 6538, *Y. enterocolitica* and *S.sonnei* followed by *L. fermentum* while *B. longum* subsp. *longum* showed no activity against *S. aureus* ATCC 6538, weak activity against *Y. enterocolitica* and good activity against *S. sonnei. L. lactis* subsp. *lactis* CFCS showed weak activity against the tested strains (Tables 3 and 4).

The tested *Lactobacillus* species (culture and CFCS) showed excellent activity against the tested *C. koseri. B. longum* subsp. *longum* culture exhibited weak activity against 2 strains of *C. koseri,* while CFCS of *B. longum* subsp. *longum* showed good activity against the tested strains. On the other hand, *L. lactis* subsp. *lactis* culture showed no activity against *C. koseri* but its cell-free extract showed good activity against 2 strains of the tested *C. koseri* strains (Table 5).

Many authors reported the production of bacteriocin-like compounds by different species of *Lactobacilli* that exhibit broad activities against Gram-positive and Gram-negative bacteria (Coconnier et al., 1997; Silva et al., 1987; Ocaña et al., 1999; Schillinger et al., 1996). The antibacterial activity of *L. paracasei* was reported by Bendali et al. (2011) as they found that *L. paracasei* showed high activity against *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Bacillus cereus*, *S. aureus* and *Enterococcus faecalis*. Also, in a study done by Ashokkumar et al. (2011), it was found that *L. paracasei* strain, isolated from donkey milk, showed a maximum activity against *E. coli* and optimum activity against *S. aureus.*

The results of this study show that *B. longum* showed activity lesser than that shown by the tested *Lactobacillus* spp. It showed activity against

Table 3. Effect of culture suspension and cell-free culture supernatant (CFCS) of LAB on the growth of *S. aureus* ATCC 6538.

LAB	Pathogens	
	***S. aureus* ATCC 6538**	
	LAB culture*	**CFCS****
L. fermentum	29±0.46	++++
L. helveticus	16±0.55	++
L. paracasei	30±0.36	+++
B. longum subsp. *longum*	-	NE
L. lactis subsp. *lactis*	-	NE

*Inhibition zones ± standard deviation (SD) in mm. **Culture supernatant was used for investigating antimicrobial activity. Diameters of inhibition zone are the mean of three replicates+ diameter of inhibition zone < 2 mm, ++ diameter of inhibition zone between 2 and 7 mm, +++ diameter of inhibition zone between 8 and 12 mm, ++++ diameter of inhibition zone between 13 and 17 mm NE no effect detected.

Table 4. Effect of culture suspension and cell-free culture supernatant (CFCS) of LAB on the growth of *Yersinia entercolitica* and *Shigella sonnei*.

LAB	Pathogens			
	Yersinia enterocolitica		***Shigella Sonnei***	
	LAB culture*	**CFCS****	**LAB culture***	**CFCS****
L. fermentum	27±0.8	+++	33±0.63	++++
L. helveticus	21±0.82	+++	29±1.1	+++
L. paracasei	32±1.4	+++	40±0.26	++++
B. longum subsp. *longum*	5±0.5	++	13±0.2	++
L. lactis subsp. *lactis*	-	NE	-	++

*Inhibition zones ± standard deviation (SD) in mm. **Culture supernatant was used for investigating antimicrobial activity. Diameters of inhibition zone are the mean of three replicates+ diameter of inhibition zone < 2 mm, ++ diameter of inhibition zone between 2 and 7 mm, +++ diameter of inhibition zone between 8 and 12 mm, ++++ diameter of inhibition zone between 13 and 17 mm NE no effect detected.

Table 5. Effect of culture suspension and cell-free culture supernatant (CFCS) of LAB on the growth of *Citrobacter koseri*.

LAB	Pathogens					
	***Citrobacter koseri* (C1)**		***Citrobacter koseri* (C2)**		***Citrobacter koseri* (C3)**	
	LAB culture*	**CFCS****	**LAB culture***	**CFCS****	**LAB culture***	**CFCS****
L. fermentum	50±1.6	+++	19±0.66	+++	20±0.96	++++
L. helveticus	32±0.93	++++	19±1.4	+++	21±0.77	+++
L. paracasei	38±0.75	++++	29±1.2	++++	30±0.45	++++
B. longum subsp. *longum*	8±0.4	++	-	++	3±0.23	++
L. lactis subsp. *lactis*	-	NE	-	++	-	++

*Inhibition zones ± standard deviation (SD) in mm. **Culture supernatant was used for investigating antimicrobial activity. Diameters of inhibition zone are the mean of three replicates+ diameter of inhibition zone < 2 mm, ++ diameter of inhibition zone between 2 and 7 mm, +++ diameter of inhibition zone between 8 and 12 mm, ++++ diameter of inhibition zone between 13 and 17 mm NE no effect detected.

E. coli, *Y. enterocolitica, S. sonnei* and *C. koseri.* Kailasapathy and Chin (2000) reported that the main therapeutic and health benefits of *L. acidophilus* and bifidobacteria are: (i) enhancement of immunity against intestinal infections, (ii) immune enhancement, (iii) prevention of diarrheal diseases, (iv) prevention of colon cancer, (v) prevention of hypercholesterolaemia, (vi) improvement in lactose utilization, (vii) prevention of

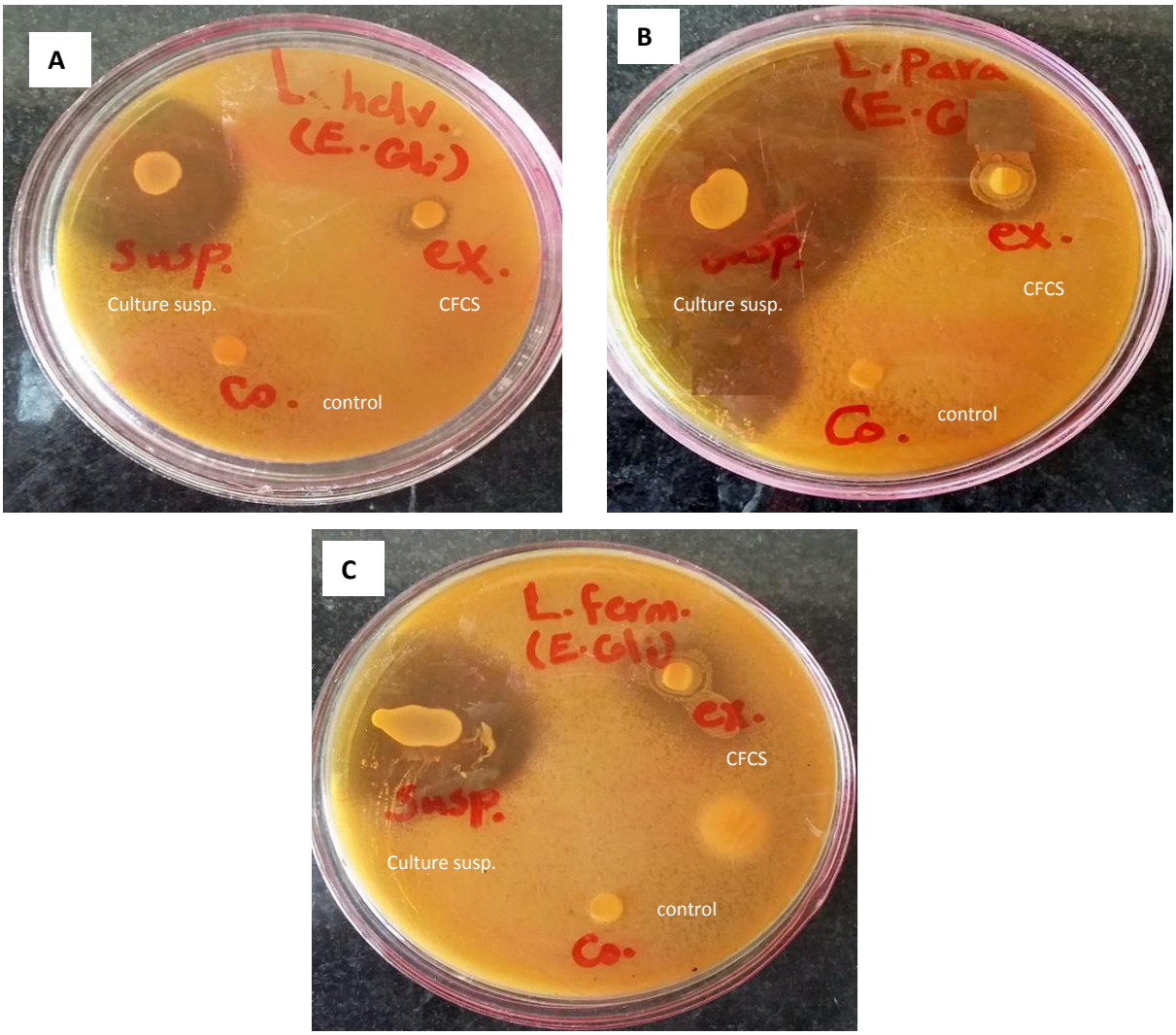

Figure 1. Antimicrobial activity of the culture suspension and the cell-free culture supernatants (CFCS) of *L. helveticus* (a), *L. paracasei* (b) and *L. fermentum* (c) against *E. coli* strain.

upper gastrointestinal tract diseases, and (viii) stabilization of the gut mucosal barrier. *Lactobacillus acidophilus* and bifidobacteria exert antagonistic effects on the growth of pathogens such as *S. aureus, Salmonella typhimurium, Y. enterocolitica* and *Clostridium perfringens* (Gilliland and Speck, 1977; Oezbas and Aytac, 1995), but in this study, *B. longum* subsp. *longum* showed no activity against *S. aureus.* In addition, *B. longum* (human origin) was found to stabilize the digestive system (Samona and Robinson, 1994).

L. helveticus is one of *Lactobacillus* strains that obtained from fermented foods and it was found to have technological importance and health promoting properties (Taverniti and Guglielmetti, 2012). *L. helveticus* can display efficient epithelium adhesion and pathogen inhibition in body sites other than the gut as, it was found that *L. helveticus* strain MIMLh5, isolated from Grana Padano natural whey starter, adheres deficiently to both, the human hypopharyngeal epithelial cell line Fa Duand HaCat keratinocytes, and inhibited the adhesion of *Streptococcus pyogenes* (the etiological agent of numerous diseases, including sore throat and acute rheumatic fever) better than the 10 probiotic and dairy lactic acid bacterial strains tested (Guglielmetti et al, 2010).

L. fermentum is one of the most important *Lactobacillus* species that have been identified in the gastric microbiota. Singh et al. (2013) reported that *L. fermentum* SBS001 of marine source showed maximum inhibitory activity to the human pathogens such as *S. aureus, K. oxytoca*, *Pseudomonas aeruginosa* and *E. coli* and minimum towards *Salmonella paratyphi*, *Proteus mirabilis*, *Vibrio cholerae* and *K. pneumoniae.*

Many studies reported that *L. lactis* has a probiotic

activity that agree with our results and has antagonistic activity against some food-borne pathogens as *Listeria monocytogenes* and *Enterococcus feacalis* (Furtado et al., 2014). Our results show that *L. lactis* subsp. *lactis* CFCS showed weak activity against the tested Gram-negative bacteria but no activity was shown against *S. aureus*. The antimicrobial activity of *L. lactis* DF04Mi against Gram-negative bacteria due to the production of bacteriocins was previously reported by Furtado et al. (2009).

Lactobacillus species in the human intestinal system act as a barrier to infection and contribute to the control of the enteric microbiota by competing with other microorganisms for adherence to epithelial cells and inhibiting the growth of potential pathogens. Hence, the use of probiotic strains of lactobacilli is potentially interesting both as preventive and curative agents.

Delivery of viable bifidobacteria in yoghurt to the consumers remains a problem. Insufficient survival of *Bifidobacterium* spp. in commercial and experimental products has been reported by a number of authors. However, possible areas for improvement in enhancing the survival and viability of these organisms are in the selection and use of strains that are resistant to acids, bile, and oxygen and possess better *in vivo* colonizing ability such as *B. longum.*

Conclusion

Conclusively, it was found out that *Lactobacillus* strains showed the highest antagonistic activity against the tested enteric pathogens in comparison to *B. longum* and *L. lactis* and it was concluded that *L. paracasei, L. helveticus*, *L. fermentum, B. longum* subsp. *longum* and *L. lactis* subsp. *lactis* are good candidates for further *in vivo* studies to elucidate their potential health benefits to be used as promising probiotic bacteria.

Conflict of interest

The authors have not declared any conflict of interest.

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