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Full Length Research Paper

Foliar application of *Azospirillum brasilense* in soybean and seed physiological quality

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Bacteria of the *Azospirillum* genus have considerable potential for application in agricultural systems, either in co-inoculation and foliar application to increase crop yields, due to its role in the production of phytohormones. The objective of this research was to evaluate the yield and quality of soybean seeds produced under the effect of different doses of *Azospirillum brasilense* bacteria applied to the leaves. Seeds of four soybean cultivars (Anta 82 RR®, BRS Favorita RR®, BRS 780 RR® and BRS 820 RR®) were produced in the 2013/2014 crop year in Lavras, Minas Gerais, Brazil, with application of six doses of inoculants based on *A. brasilense* (0, 300, 400, 500, 600 and 700 mL ha^{-1}) of the strains, AbV_5 and AbV_6. Besides the yield, the mass of a thousand seeds, moisture content, germination, emergence at 7 and 15 days after sowing, emergence speed index, accelerated aging, strength, viability and mechanical damage were evaluated by tetrazolium and the hypochlorite test. Regardless of the soybean cultivar, application of up to 700 $mLha^{-1}$ of *A. brasilense* innoculant at v_3 stage (second open trefoil) of the plants, the yield, physiological potential and seeds were not affected.

Key words: *Glycine max* (L.) Merrill, growth promoting bacteria, emergency, viability, vigor.

INTRODUCTION

In the last decades, the soybean crops [*Glycine max* (L.) Merrill] have expanded to several regions of the world. In this scenario, Brazil stands out in the production of this oilseed, being the second largest producer of soybeans. In 2014/2015 cropping year, the soybean crops occupied an area of 31.33 million hectares, which reached the total production of 93.25 million tons. In the Southeast, the state of Minas Gerais is the largest producer of soybeans, with an area of about 1.31 million hectares (CONAB, 2015).

In commercial crops and seed production fields, inoculation with *Bradyrhizobium japonicum* bacteria is common eliminating the use of nitrogen fertilization due to the efficiency of biological nitrogen fixation (BNF). Recently, it has been speculated that the use of *Azospirillum brasilense* bacteria can increase the soybean crops performance (Hungria et al., 2007). Bacteria of the genus *Azospirillum*, produce phytohormones,

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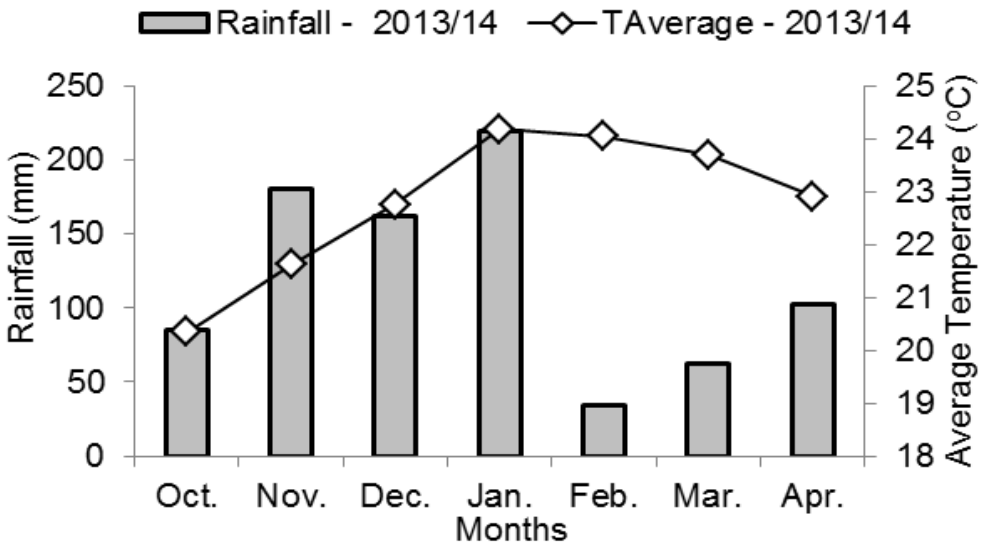

Figure 1. Monthly means for rainfall and air temperature in Lavras, MG, Brazil, in 2013/2014 crop year during soybean production. Source: National Meteorology Institute (INMET).

including auxins, gibberellins, cytokinins, that under "*in vitro*" conditions (Araujo, 2008; Masciarelli et al., 2013), can reduce the need of chemical inputs application, provide the fixing of atmospheric nitrogen, reduce biotic and abiotic stresses, and increase crop yield (Hungria, 2011). These benefits can improve the physiological potential of seeds. Cassán et al. (2009) found that in corn (*Zea mays* L.) and soybean crops, the treatment of seeds with *A. brasilense* strains led to significant increase in germination and vigor. However, in wheat crop(*Triticum aestivum* L.), Brzezinski et al. (2014) found that the sanitary quality of wheat seed is not influenced by *A. brasilense* inoculation. The inoculation of wheat seeds with *A. brasilense* favors the vigor (accelerated aging) and seedlings shoot mass.

According to Azevedo et al. (2007), little has been studied on the effects of cultural practices on the physiological quality of soybean seed. It is known that high quality seeds are desirable for the success of agriculture. Thus, the objective of the research was to evaluate the yield and quality of soybean seed produced under the effect of different doses of *A. brasilense* bacteria inoculation applied to the leaves.

MATERIALS AND METHODS

The seeds were produced in 2013/2014 crop year, in Lavras - MG, Brazil, at the Scientific and Technological Development Center of Agriculture - at UFLA, located at 21°12'S latitude, 44°58'W longitude and altitude of 918 m in soil classified as distroferric Red Latosol - LVdf.

The climate is Cwa, according to the Köppen classification, with an average annual temperature of 19.3°C and normal annual rainfall of 1,530 mm (Dantas et al., 2007). During the seed production process, the climatic data was collected at the meteorological station of the National Institute of Meteorology (INMET) located at the Federal University of Lavras-UFLA and are presented in Figure 1.

The experiment was performed in a randomized block design with three replications. The treatments were arranged in a 4 x 6 factorial, four soybean cultivars seeds were produced (Anta 82 RR®, BRS Favorita RR®, BRS 780 RR®, BRS 820 RR®) with the application of six doses of the inoculants based on *A. brasilense* (0, 300, 400, 500, 600 and 700 mL ha^{-1}) applied to the leaves in the V_3 stage (second open trefoil). Each plot consisted of four sowing rows of 5 m in length, spaced at 0.50 m, and the area of each plot was of 10 m^2 (5 m x 2 m). The two central rows were considered as useful area.

The sowing was performed in November 2013. The fertilization consisted of 350 kg ha^{-1} of N-P_2O_5-K_2O (02-30-20), applied via groove. The *Bradyrhizobium japonicum* bacteria were inoculated via groove after soybean seeding. The dose of *B. japonicum* was 18 mL p. c. kg^{-1} of seed – strains SEMIA 5079, containing 10.8 x 10^6 CFU/seed of Nitragin Cell Tech HC® (3x10^9 CFU/mL). the inoculant Azo® (1 x 10^8 CFU/mL) strains estirpes AbV_5 and AbV_6 were used. The application of microorganisms was performed using a motor-driven backpack sprayer, coupled to a bar with four spray nozzles XR 110.02, applying spray volume equivalent to 150 L ha^{-1}. The legumes were harvested manually and threshed by stationary threshing in order to simulate the mechanical harvesting held by the harvester. The grain yield (kg ha^{-1}), being standardized for grainmoisture of 13% was determined. Subsequently, the seeds were separated from impurities with the assistance of sieves, and packaged in 'Kraft' model paper bags. Seeds with moisture content above 13% were placed in the shade to slow drying. After determination of the adequate moisture content, mixing of samples and separation in sieves were done/performed. For the laboratory tests, seeds classified in the 6.00 mm diameter sieve were used. Laboratory evaluations were performed using a completely randomized design, all the tests were performed with two subsamples of 50 seeds per replication, totaling 300 seeds per treatment. The physiological and physical quality of the seeds were determined using the following determinations:

Thousand seeds mass and moisture content

Recommendation of Brasil (2009) was followed.

Germination test

This was done in the paper roll form with a water volume in the amount of 2.5 times the dry mass of the substrate at 2°C. The evaluations were performed on the 8th day after sowing, according to the criteria established by the Rules for Seed (BRASIL, 2009).

Emergence test under controlled conditions

The substrate used was composed of soil and sand (2:1). After sowing, the trains were kept in a greenhouse at 25°and relative air humidity of ±70%. After the emergence of the first seedling (visible cotyledon), daily evaluations were made for the number of emerged plants, with the final counting 15 days after sowing. For the speed emergence index (SEI) calculation, the Maguire (1962) formula was adopted.

Accelerating aging test

This was realized according to Marcos Filho (1999) recommendations, with 42 g seeds per treatment on an adapted stainless steel grille in *gerbox* boxes, with 40 mL of water in the bottom, maintained at 41°C for 72 h in a BOD and were evaluated at germination.

Tetrazolium test

The seeds were placed in a *germitest* paper moistened for 16 h at 25°C. After this period, they were submerged in 0.075% tetrazolium salt solution for three hours at 40°C in an incubation chamber. The viability, the damage percentage and the vigor were determined according to Neto et al. (1998).

Sodium hypochlorite test

For the sodium hypochlorite test, the seeds were kept for 10 min in a sodium hypochlorite solution (0.2%) and evaluated in accordance with Krzyzanowski et al. (2004).

Statistical analysis

The variance analysis was realized adopting the statistical model and the analysis procedure similar to Ramalho et al. (2012). The means were grouped by the Scott-Knott test (1974). The statistical analysis was realized with SISVAR® statistical package (Ferreira, 2011).

RESULTS AND DISCUSSION

The inoculation doses with isolated *A. brasilense* bacteria and the interaction of cultivars (C) and bacteria (A) did not lead to a significant difference on the studied tests (Table 1). According to this fact, it can be inferred that the physiological quality of the soybean seeds, on average, does not depend on the *A. brasilense* inoculation dose and on the studied cultivar. Similar results were also obtained by Zuffo et al. (2015a) and Zuffo et al. (2015b). The authors did not observe the *A. brasilense* bacteria on the soybean agronomical characters.

A possible explanation for this fact is that the used doses did not promote any effect on the physiological quality because of the absence of effect on agronomical characters, as verified by Gitti et al. (2012), Zuffo et al. (2015a, b). With no influence on agronomical characteristics on the field, the plant produced seeds with a similar physiological potential. On the other hand, when different seeds from different cultivars were evaluated, differences on the physiological quality were observed (Table 1). The cultivar effect on the soybean seed physiological quality was verified by Zambuzzi et al. (2014). The cultivars have different characteristics related to the genetic background, growth habit, maturation group and other attributes, promoting the existence of some variations.

For cultivars, a high amplitude between the means for thousand seed mass was observed, from 187.22 g to 127.77 g for BRS Favorita RR® and Anta 82 RR®, respectively (Table 1), however, all the cultivars presented satisfactory productive performance, with results above the average mean for the crop in Minas Gerais state – 2658 kg ha^{-1} – achieved during the 2014/2015 crop year (CONAB, 2015).

The moisture contents of seeds from different cultivars were similar (Table 1). According to Leoffler et al. (1998), the uniformity on the moisture content is very important to standardize the evaluations and provide consistent results.

In a general manner, the studied cultivars presented a lower germination percentage as compared to the required standard to commercialize seeds in Brazil, which is 80%, established by the normative instruction no. 45 (BRASIL, 2013). Seeds with low or medium germination can generate less competitive seedlings in the field (Neto et al., 2010). The main reason for the low germination can be related to the higher percentage of dead and infected seeds verified during the germination test (Figure 2). According to Binotti et al. (2008), the seed pathogens can increase the deterioration and reduce the vigor and germination of the seeds. During the emergence tests, 7 and 15 days after emergence, higher means were observed as compared to germination. The cultivars Anta 82 RR® and BRS 780 RR® demonstrated more emergence percentage. The higher means from emergence test, when compared with germination test have already been mentioned in literature by Henning and Neto (1980), Bizzetto and Homechin (1997) and Zambiazzi et al. (2014). The authors related that the seedlings, on emergence, releases the infected tegument on the soil, but during the germination test, on the paper, the tegument remains associated with the cotyledons and the fungus promote the seed deterioration.

For the speed emergence index, the same trend related to the emergence test was observed. This fact

Table 1. Mean values for yield, thousand seeds mass (TSM), moisture content (MC), germination (GERM), emergence 7 days after emergence – DAE (E7DAE), emergence 15 DAE (E15DAE), speed emergence index (SEI), accelerated aging (AAG), vigor percentage by tetrazolium ($Tz_{(1-3)}$), viability percentage by tetrazolium ($Tz_{(1-5)}$) and damage percentage by hypochlorite (HYPO) obtained in tests with different doses of inoculant with *Azospirillum brasilense* bacterias applied at V_3 stage in soybean cultivars during the 2013/2014 crop year. Lavras, MG, Brazil.

Source of variation	Yield (kg ha^{-1})	TSM (g)	MC (%)	GERM (%)	E7DAE (%)	E15DAE (%)	SEI (%)	AAG (%)	$Tz_{(1-3)}$ (%)	$Tz_{(1-5)}$ (%)	HYPO (%)
Cultivar (C)											
Anta 82 RR®	3262^{c}	127.77^{c}	8.27^{a}	62^{a}	90^{a}	93^{a}	72.61^{a}	79.38^{a}	93.03^{a}	96.93^{a}	7.00^{b}
BRS Favorita RR®	4202^{a}	187.22^{a}	8.43^{a}	65^{a}	86^{b}	88^{b}	69.32^{b}	61.38^{c}	88.47^{a}	95.56^{a}	11.22^{a}
BRS 780 RR®	3814^{b}	168.88^{b}	8.39^{a}	59^{b}	92^{a}	94^{a}	75.69^{a}	72.00^{b}	92.55^{a}	96.81^{a}	6.98^{b}
BRS 820 RR®	4385^{a}	164.88^{b}	8.58^{a}	59^{b}	88^{b}	89^{b}	70.54^{b}	44.16^{d}	89.51^{a}	95.26^{a}	10.94^{a}
P (Value)	<0.01**	<0.01**	0.10^{ns}	<0.01**	<0.01**	<0.01**	<0.01**	<0.01**	0.12^{ns}	0.53^{ns}	<0.01**
Azospirillum brasilense (A) (mL ha^{-1})											
0	4037	160.00	8.46	63	88	90	71.91	63.25	90.53	97.52	8.87
300	3784	161.66	8.54	60	89	91	71.88	66.66	88.09	94.91	8.91
400	4003	165.00	8.39	58	87	91	69.16	64.33	93.22	97.82	8.87
500	3903	157.50	8.37	61	91	92	73.25	59.83	91.98	95.80	9.00
600	3969	170.00	8.37	62	90	91	71.98	65.41	90.64	94.90	9.41
700	3800	165.00	8.38	60	90	92	74.06	65.91	90.86	95.89	9.14
P (Value)	0.30^{ns}	0.66^{ns}	0.87^{ns}	0.12^{ns}	0.32^{ns}	0.86^{ns}	0.18^{ns}	0.24^{ns}	0.56^{ns}	0.38^{ns}	0.83^{ns}
C x A	0.11^{ns}	0.30^{ns}	0.44^{ns}	0.07^{ns}	0.61^{ns}	0.52^{ns}	0.64^{ns}	0.06^{ns}	0.88^{ns}	0.77^{ns}	0.79^{ns}
CV (%)	8.39	11.65	4.49	7.05	5.27	4.98	6.38	11.31	7.38	4.36	12.61

**Significant at 1% according to F test, ns – not significant. Means followed by the same lower case in the column are from the same group, according to Skott Knott test (1974) at 5% of probability.

was expected because the seedlings that emerge first can grow more and have more biomass because of photosynthesis in the first growth stages. Besides this, in field conditions, the seedlings from seeds with a higher speed emergence index promote a faster closure between the field lines, leading to better weed control (França et al., 2010). For the accelerating aging test, the cultivar BRS 820 RR® followed by BRS Favorita RR® was more sensitive to high temperature and relative humidity conditions. For the vigor percentage and seed viability, measured by tetrazolium, no significant difference between cultivars was observed. For the mechanical damages, moisture deterioration and stink bug damage were found to be the main problems for the soybean; different damages were observed according to cultivar (Figure 3). Thus, cultivars have distinct genetic characteristics that can lead to higher or lower susceptibility to damages from bugs, harvest or adverse conditions. For the damage percentage by hypochlorite, Anta 82 RR® and BRS 780 RR® presented lower means. This data support the results obtained for mechanical damages by tetrazolium, demonstrating consistency between the tests, but without observing effect of different doses of inoculant with *A. brasilense* on the physical and chemical seed quality.

Conclusion

Despite the soybean cultivar, the inoculant application with *A. brasilense* bacteria up to 700 mL/ha applied on plants at V_3 stage did not affect the yield, physiological potential and seed

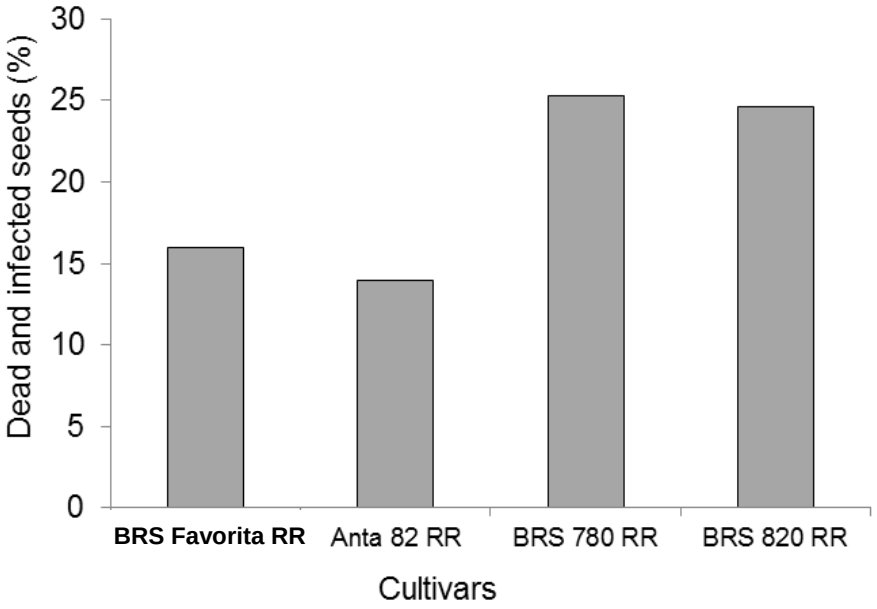

Figure 2. Total percentage of dead and infected seeds during germination test for each cultivar during the 2013/2014 crop year. Lavras. MG. Brazil.

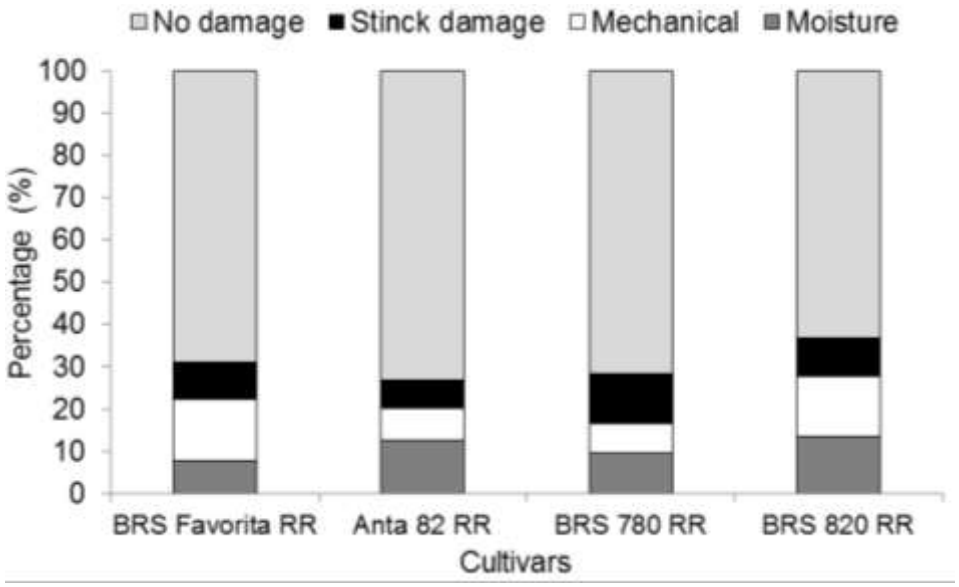

Figure 3. Damages and their respective percentages (%) observed during tetrazolium test for each cultivar during the 2013/2014 crop year. Lavras. MG. Brazil.

damage incidence.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Antibacterial activity of extracts of three aromatic plants from Burkina Faso against rice pathogen, *Xanthomanas oryzae*

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Plant extracts can have powerful antibacterial activity and may be used as biological control against important plant pathogens. This study assessed effect of extracts of *Cymbopogon citratus*, *Eucalyptus camaldulensis* and *Mentha piperita* against two pathovars of *Xanthomonas oryzae* attacking rice. Both *in vitro* and *in vivo* approaches were used for different strains of *X. oryzae* originating from Burkina Faso. The three essential oils (EOs) have antibacterial activity *in vitro* test (agar diffusion method), but, EO from *C. citratus* at 1:5 dilution (v/v) resulted in the highest inhibition (over 30 mm of inhibition zone) against *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*. However, they reduced significantly rice seed germination and induced herbicide effects on rice leaves. On the other hand, aqueous extracts from *C. citratus* stimulated bacterial growth, while extracts from *E. camaldulensis* had an inhibitory effect (28 mm of inhibition zone at 3:10 dilution, w/v). Therefore, the characterization of active compounds will determine one or more compound(s) involved in the antibacterial activity.

Key words: *Xanthomonas oryzae*, rice, plant extracts, antibacterial activity.

INTRODUCTION

Rice (*Oryza sativa*) is the third most important cereal crop in the world following wheat and maize with an estimated production of 500 million metric tons in 2015 (www.fao.org). It is a staple food in developing countries and its consumption is steadily increasing. In Burkina Faso, the average per capita consumption of rice is about 18 kg per year. However, rice production faces several important constraints including rice diseases. Providing healthy rice seeds is a requirement to increase rice productivity in order to answer the challenge of feeding fast growing populations. Indeed, several seed-borne bacterial pathogens of rice were described, and among those, *Xanthomonas oryzae* causes important yield losses. Bacterial leaf blight (BLB) and bacterial leaf streak (BLS) are two important diseases due to *X. oryzae* pv. *oryzae* (*Xoo*) and *X. oryzae* pv. *oryzicola* (*Xoc*), respectively; they significantly reduce global rice production in the tropical areas. BLB is present in tropical

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and temperate areas wherever rice is grown. The yields losses induced by BLB under irrigation scheme can reach about 20 to 80% depending on the rice variety and climatic conditions (Ou, 1985). However, BLS is mostly confined to tropical and subtropical areas in Asia, Africa and Australia (Ou, 1985) and can cause yield losses of up to 30% (Gonzalez et al., 2007). BLS was first reported in Burkina Faso in 2009 by Wonni et al. (2011). The disease is present in all major rice production areas and it is becoming a threat to rice production (Wonni et al., 2014).

Seeds are primary sources of inoculum for both bacterial diseases (Ou, 1985; Mew et al., 1993; Agarwal et al., 1994). *Xoo* and *Xoc* are quarantine pathogens in the USA and in some countries where these pathogens are endemic in order to limit the introduction of new virulent strains.

To avoid the dissemination of bacterial diseases by the means of infected seeds, Agarwal et al. (1994) suggested seeds disinfection with bactericides and heat. However, chemical treatments are expensive for the small rice producers of developing countries and harmful for environment and human health. Also, the thermotherapy needs a device that can maintain a constant temperature duration of treatment. The lack of seeds treatment against *X. oryzae*, and consequently the exchange of contaminated seeds, are likely involved in the rapid range expansion of BLB and BLS.

Compounds of some tropical aromatic plants have been reported to possess potent antimicrobial activities (Ilodibia et al., 2015; Akale et al., 2015; Singh et al., 2016; Salem et al., 2016). Notably, antibacterial activity was reported for three plants found commonly in Burkina Faso: *Cymbopogon citratus*, *Eucalyptus camaldulensis* and *Mentha pipera*.

C. citratus or lemongrass is a perennial tropical grass; it is resistant to different temperatures and can grow in all climates. The essential oil from *C. citratus*, besides having antimicrobial effects, has been shown to have anti-inflammatory, analgesic and antipyretic properties (Kpoviessi et al., 2014; Vázquez-Briones et al., 2015). *Mentha piperita* L., a medicinally important plant belonging to the family Lamiaceae and commonly known as peppermint is a hybrid of *Mentha spicata* L. and *Mentha aquatic.* The extracts of *Mentha piperita* are found to possess antibacterial, antiviral and antifungal activities (Singh et al., 2016; Ilboudo et al., 2016). The leaves of *Eucalyptus camaldulensis* are usually used for various purposes, including treatment of infections. All these plants are grown traditionally on small or large areas everywhere in Burkina Faso, so interesting candidates for biological control of bacterial diseases.

The present study aimed at assessing antibacterial activities of extracts from three aromatic plants (*C. citratus*, *E. camaldulensis* and *M. pipera)* on *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* strains *in vitro* and to assess their potential antibacterial effect on rice seed germination and rice plant development.

MATERIALS AND METHODS

Bacterial cultures

The authors used two strains of *X. oryzae* originating from Burkina Faso: BAI3 (*X. oryzae* pv. *oryzae*) isolated from rice plants with BLB in 2003 by Gonzalez et al. (2007) and BAI105 (*X. oryzae* pv. *oryzicola*) isolated from BLS symptoms in 2012 by Wonni et al. (2014). These strains belong to major group representing the genetic diversity in West Africa (Poulin et al., 2015). These strains from INERA-IRD collection were stored at -80°C and recovered on Peptone, Sucrose, Agar (PSA) medium for use.

Vegetal material source

The leaves of *C. citratus*, *E. camaldulensis* and *M. piperita* were collected in the station of environmental and agricultural research of Farako-Ba located 10 km from the city of Bobo Dioulasso (11°9'56.97''N; 4°18'19.80''W) in Burkina Faso. After collection, all the others tests were achieved in the laboratory.

Preparation of aqueous extracts for phytochemicals analysis

Leaves were dried at 40°C and milled to obtain a fine powder using a vegetable blender and then stored in airtight bottles. 100 g of fined powder of each plant was suspended in 100 ml of distilled water and kept under room temperature for 24 h. Then, it was filtrated through muslin cloth and centrifuged at 5000 rpm for 15 min. The supernatant was collected under vacuum using a millipore membrane of 0.20 µm. The aqueous extract obtained was stored at 4°C for further use. Dilutions of 1:10; 2:10 and 3:10 were used for antibacterial activity tests.

Preparation of essential oils for phytochemicals tests

Essential oils (EOs) from *C. citratus*, *E. camaldulensis* and *M. piperita* were obtained from Phytofla laboratory located in Banfora (10°38'26.09''N; 4°45'33.15''W), a town located in the west of Burkina Faso. These EOs were extracted from leaves by the hydrodistillation method. The emulsion of EOs and 0.1% water-agar solution were prepared following Remmal et al. (1993) method. Each EOs was mixed at the ratio of 1:5, 1:10 and 1:20 (v/v).

In vitro antibacterial assay

The modified method of Nguefack et al. (2005) was used to study the antibacterial activity of plants extracts. 15 µl of bacterial suspension concentrated at 10^8 CFU was inoculated in 4.5 ml of PSA liquid medium at 75%. The mix was homogenized and poured in solid PSA medium containing plates.

A volume of 10 µl of each ratio of emulsion oils and aqueous extract respectively was placed equidistantly onto *Xoo* and *Xoc* spread medium followed by incubation at 28°C. Water-agar was used as the control treatment. The inhibition zones were measured after 96 h of incubation. Each treatment was replicated three times, with three plates per replication. Three independent assays were performed with similar results.

In vivo antibacterial assay

The EOs from *C. citratus* and *E. camaldulensis* respectively found

Table 1. Mean diameter (mm ±SE) of inhibition zone of *Xanthomonas oryzae* strains with essential oils from *Cymbopogon citratus, Eucalyptus camaldulensis* and *Mentha piperita*.

Essential oils	Mean diameter of inhibition area					
	Ratio 1:5		Ratio 1:10		Ratio 1:20	
	Xoo	*Xoc*	*Xoo*	*Xoc*	*Xoo*	*Xoc*
C. citratus	31±1[b]	34.33±0.00[c]	26±0.76[b]	29.55±3.50[b]	18±1[b]	13.34±0.58[c]
E. camaldulensis	13.78±1.7[a]	12.67±1.34[b]	9.67±0.88[a]	9.44±0.20[a]	8±0.00[a]	8.67±0.34[b]
M. piperita	12.78±0.39[a]	10.22±1.17[a]	8.67±0.00[a]	7.67±0.58[a]	8.11±0.19[a]	6.56±0.20[a]

Values followed by different letters are significantly different (P< 0.05) based on Duncan's multiple range test.

to be highly and moderately effective in suppressing the growth of *Xoo* and *Xoc* strains *in vitro*, were tested for their antibacterial activity on seed germination and their effects on rice leaves.

Effect of EOs on rice seeds germination

The seeds were treated with an emulsion of EOs from *C. citratus* and *E. camaldulensis,* and with agar-water at the following ratios: 1:5, 1:10, 1:20 and 1:25. 100 µl of each EO mixture was applied on 400 rice seeds according to Adegoke and Odela (1996) method. The treated seeds were placed at room temperature for 24 h before germination test. One control with water-agar solution without EOs was used. Then, the seeds treated were tested using the blotter papers method. Four replicates of 100 seeds for each EO were rolled in blotter papers and placed in polyethylene bags then incubated at 28-30°C under a cycle of 12 h light/12 h darkness. The rate of seeds germination was assessed at 3, 5 and 7 days after incubation. The experiment was repeated twice.

Effect of EOs on rice leaves

The effect of EOs on leaves was tested for efficient concentration of *C. citratus* and *E. camaldulensis* at the concentration found to have antibacterial effect *in vitro*. Three EOs emulsions at the ratio of 1:5; 1:10 and 1:20 were sprayed on both sides of each leaf from thirty days old plants. Five leaves were treated with each EOs and three replicates were done. Data were collected on the leaves 48 h after treatment.

Statistical analysis

The data on diameter of inhibition area and the germination rate of rice seeds treated, induced by the essential oils were assessed by analysis of variance (ANOVA), and treatment means were compared by Duncan's multiple range test. Statistical significance was set at P<0.05 and the analyses were performed with IBM SPSS Statistics Base 20 software.

RESULTS

Effect of plant extract *in vitro*

Efficiency of essential oils

All tested EOs exhibited considerable antibacterial activity against both pathovars of *X. oryzae* (Table 1). EOs from *C. citratus* was the most effective in inhibition

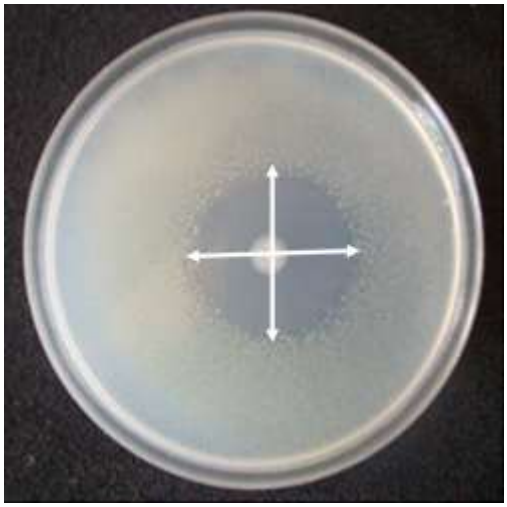

Figure 1. Inhibition zone (34 mm of diameter) of the growth of *X. oryzae* pv. *oryzae* induced by essential oil from *C. citratus* at the ratio 1:5.

of *Xoo* and *Xoc* growth than *E. camaldulensis* and *M. piperita*. The inhibition area of *Xoc* at 1:5 ratio was to 34 mm for OE from *C. citratus* (Figure 1) against 12.67 and 10.22 mm OEs from *E. camaldulensis* and *M. piperita* respectively.

Efficiency of aqueous extracts

Bacterial growth was affected by the aqueous extract of *E. camaldulensis* only with 19.67 and 15 mm mean diameter of inhibition zone, respectively for *Xoc* and *Xoo* after 5 days incubation at 1:10 dilution (data not shown). The inhibition effect increases with the high concentration of aqueous extracts. In comparison with the control, aqueous extract from *C. citratus* stimulates the growth of *X. oryae* strains at the level of the deposit point of the drop of aqueous extracts (Figure 2).

In vivo activity of essential oils

Effect of EOs on rice leaves

The three efficient concentrations of EOs from *C. citratus*

Figure 2. Stimulation of the growth of *Xanthomonas oryzae* pv. *oryzicola* strain in deposit area of aqueous extracts of *Cymbopogon citratus.*

Figure 3. Effect of essential oils of *Cymbopogon citratus* and *Eucalyptus camaldulensis* on rice leaves.

and *E. camaldulensis* used to treat rice leaves *in vitro* induce partial burns or complete desiccation of the leaves (Figure 3).

Effect of EOs on rice seeds germination

The rate of seeds germination varied according to EOs and the incubation duration. The seeds treated with Eos from *E. camaldulensis* and *C. citratus* at the ratio of 1:5 and 1:10 reduced significantly seed germination (≤70%) as compared to the control. However, at the concentration of 1:20, the percentage of germination was similar to the control (Figure 4).

DISCUSSION

EOs from *C. citratus* was found to be highly effective in inhibiting the growth of two pathovars of *X. oryzae*. Moderate activity was recorded from Eos of *E. camaldulensis* and M. piperita. The data showed that efficiency of each EOs was similar for both pathovars of *X. oryzae*. Considering aqueous leaf extracts, it was found that only *E. camaldulensis* inhibited *Xoo* and *Xoc* growth, while *C. citratus* stimulated bacterial growth as compared to the control.

The efficiency of extracts from the three aromatic plants on phytopathogenic bacteria was previously reported (Nguefack et al., 2005; Paret et al., 2010; Lucas et al., 2012). In addition, several plant extracts are known to possess antibacterial activity. Govindappa et al. (2011) have reported antibacterial activity of aqueous extract of *Adathoda vasica* on *Xoo*. Nguefack et al. (2005) demonstrated the efficiency of EOs from *Ocimum gratissimum* and *Thymus vulgaris* on *Xoc* NCPPB 1632 R1 strain. In contrast, they found that *Xoo* NCPPB 2446R11 strain was not sensitive to EO from *C. citratus* at 1:10 dilution which is not in conformity with the present study results.

The antibacterial activity results from the chemical composition of each essential oil. EOs from *C. citratus* contains 80% of citral and *E. camaldulensis* contains 77% of 1,8-cinéol which give them antibacterial properties (Mehani and Ladjei, 2012; Vázquez-Briones et al., 2015). Singh et al. (2016) reported the strong antibacterial effect of *M. piperita*. It contain active ingredients such as flavonoids, polymerized polyphenols, carotenes, tannins, etc; known to play antibacterial activity (Sokovic et al., 2009).

The results showed that seeds treatment with the EOs from *C. citratus* and *E. camaldulensis* reduces seeds germination rate, except when applied at a low concentration. Also, these EOs cause various symptoms ranging from burns to drying, when applied on the leaves. The secondary metabolites contained in the EOs of these two aromatic plants could explain the reduction of seeds germination rate, burns and leaves drying that was observed following the treatment and may confer them allelopathic and herbicide effects. Indeed, Gargouri et al. (2014) reported that *C. citratus* and *E. camaldulensis* exhibited inhibitory effects on associated vegetation. It is known also that the use of citrals-rich EOs induces the inhibition of weed seeds germination. For instance, the citrals, major component of the EO from *C. citratus* showed phytotoxicity effects against *Sinapis arvensis* and *Phalaris canariensis* even at low concentration.

During this study, the antibacterial activity of the EOs from *C. citratus*, *E. camaldulensis* and *M. piperita* against both pathovars of *X. oryzae* was demonstrated. These plants are an alternative to the use of pesticides and have the advantage of protecting human health and the environment. However, these results reveal that the effective concentrations of these Eos reduce seeds

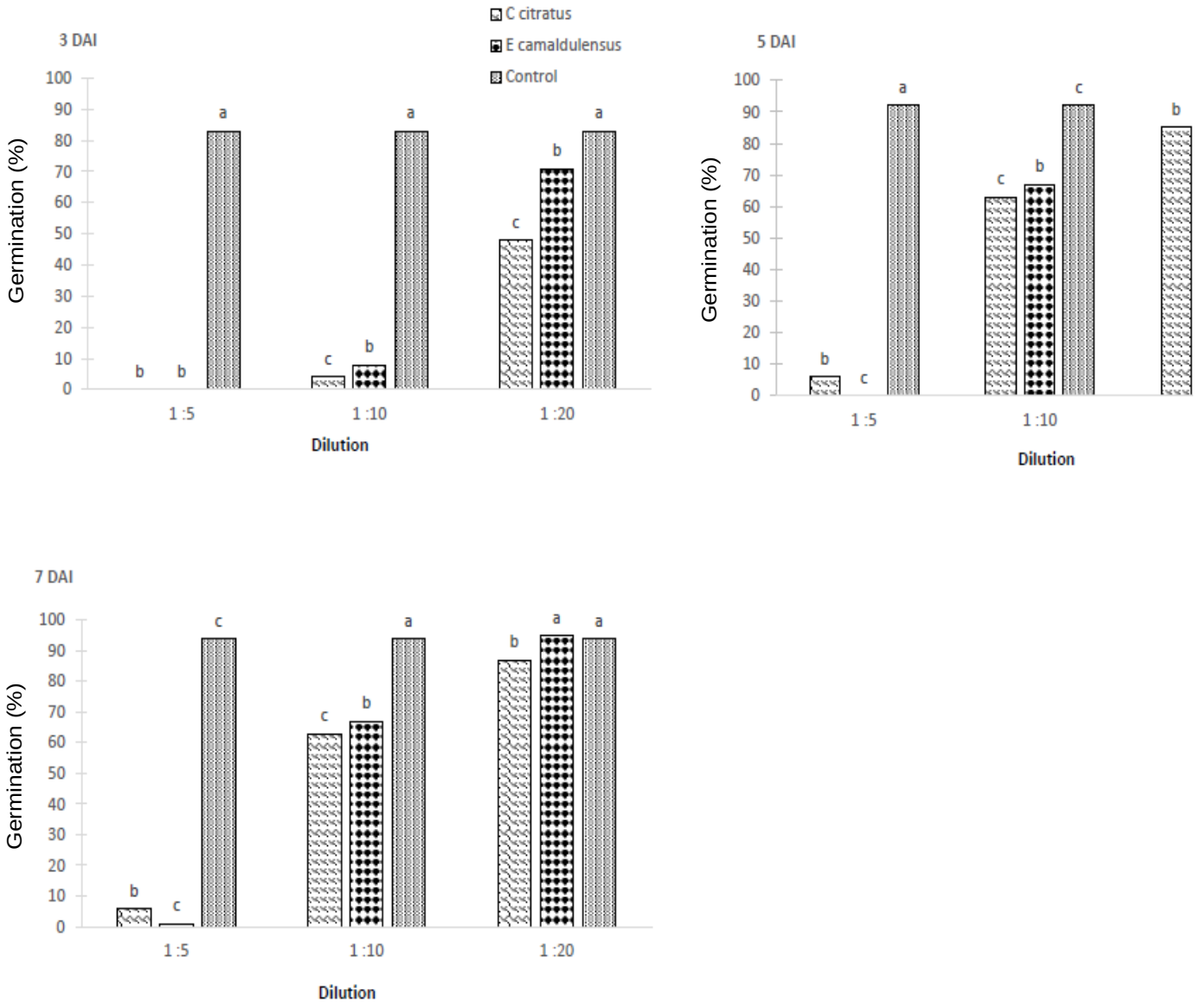

Figure 4. Germination rate of Azecuna seeds treated with essential oil from *Eucalyptus camaldulensis* and *Cymbopogon citratus*. The germination rate was evaluated at 3, 5 and 7 days after incubation (DAI). Different letters denote a significant difference between essential oil at 1:5, 1:10 and 1:20 dilution.

germination and induce herbicide effects on rice leaves.

This study shows the identification of molecules responsible for the antibacterial activity and their purification in order to test their effects on seeds contaminated with *Xoo* and *Xoc*. Also, the components of aqueous extracts from *E. camaldulensis* that stimulates *Xo* strains growth could be used to enrich medium culture.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Effect of tyrosol on *Staphylococcus aureus* antimicrobial susceptibility, biofilm formation and virulence factors

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Staphylococcus aureus **is considered the most common causative agent of community and hospital-acquired infections. The isolates with increased virulence and antimicrobial resistance, especially mecithillin resistant *S. aureus* (MRSA), represent a challenge in hospitals and health care facilities worldwide. The effect of tyrosol, a phenolic quorum sensing compound of *Candida albicans*, was studied on the sensitivity of clinical MRSA isolates to antibiotics. Besides having antibacterial activity, subinhibitory concentrations (3.5 - 14.3 mM) of tyrosol increased the susceptibility to antimicrobial. It gave either synergistic or additive effect when combined with gentamicin, amikacin and ciprofloxacin. Also, the effect of such concentrations on virulence factors production was investigated. Biofilm formed was significantly decreased in most of the tested isolates (P≤0.0001). In addition, it significantly decreased the production of protease and lipase enzymes. Overall, these results represent a promising method for inhibiting *S. aureus* either by reducing its resistance to antibiotics or decreasing the production of virulence factors.**

Key words: *Staphylococcus aureus*, tyrosol, antibiotic sensitivity, virulence factors.

INTRODUCTION

Staphylococcus aureus is a commensal organism that can asymptomatically colonize healthy individuals. Also, it is one of the important pathogens of both hospital and community acquired infections (Gordon and Lowy, 2008; Plata et al., 2009). This pathogen can cause a wide range of infections which may be superfacial, systemic or toxin mediated (Kluytmans et al., 1997; Schito, 2006).

Shortly after the first introduction of methicillin in 1960, methicillin resistant *S. aureus* isolates were reported. MRSA is characterized by the presence of *mecA* gene that results in decreased affinity for β- lactam antibiotics as it encodes penicillin binding proteins 2A(PBP2A) (Gordon and Lowy, 2008). MRSA is considered a major problem worldwide as it results in worse consequences such as prolonged time of hospitalization, increased cost of treatment and mortality (Onelum et al., 2015). The

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increasing prevalence of MRSA requires the development of new treatment strategies for serious infections which became an urgent matter (Shah, 2005).

S. aureus possesses an arsenal of virulence determinants that promote tissue colonization, invasion, avoiding the immune systems and dissemination of the disease (Bien et al., 2011). These determinants include adherence factors (adhesins) and secreted products (exoproteins). Adhesins are involved in attachment and initiation of colonization. The organism can reside in biofilm in prosthetic materials causing its persistence and escaping the host defense and antimicrobials (Donlan and Costerton, 2002). Exoproteins such as exotoxins and enzymes (lipases, proteases, hyaluronidase, hemolysins and nucleases) are produced during infection to help in invasion and destruction of tissues (Dinges et al., 2000).

Tyrosol (2-(4-Hydroxyphenyl) ethanol) is a known phenolic compound which is a quorum-sensing molecule of *Candida albicans*. Similar to farnesol, tyrosol is released into the growth medium and accelerates the formation of germ tubes (Alem et al., 2006). Therefore, it appears that tyrosol and farnesol control positively and negatively the morphogenesis in *C. albicans*.

Moreover, it has antioxidant properties and scavenging effects on reactive oxygen and nitrogen species that are implicated in human pathologies such as cardiovascular and thrombotic diseases. It is found in food sources such as olive oil, olive leaves and green tea (Miro-Casas et al., 2003; Romero and Brenes, 2012) and terrestrial fungi. Tyrosol also showed antimicrobial activity (Alem et al., 2006; Guimarães et al., 2009).

Therefore, this study aims to evaluate the effect of tyrosol on the antimicrobial sensitivity of clinical MRSA isolates, in addition to its effect on biofilm and production of some virulence factors.

MATERIALS AND METHODS

Bacterial isolates and reagents

In this study, clinical isolates of *S. aureus* (MRSA) were isolated from Mansoura University Hospitals. MRSA was identified according to sensitivity to cefoxitin disc (CLSI, 2014) and presence of *mecA* gene by PCR (Kondo et al., 2007). They were stored at -70°C. Tyrosol and p-nitrophenylpalmitate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Mueller-Hinton broth (CAMHB, Sigma-Aldrich), LB broth and egg-yolk tellurite emulsion were obtained from Oxoid. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was obtained from SERVA Electrophoresis GmbH (Germany).

Determination of MIC

The minimal inhibitory concentrations (MICs) of tyrosol, gentamicin, amikacin and ciprofloxacin against *S. aureus* isolates were determined by the broth microdilution method according to CLSI (2014). Briefly, two fold serial dilutions of each tested drug in 96 well microtitreplate were inoculated by cultures of tested isolates. Positive and negative controls were included in each plate. The microtitre plate was incubated at 37°C, the endpoint was read spectrophotometrically using microplate reader (BioTek ELx800) after 24 h and the MIC was determined.

Determination of the combined effect of tyrosol and antibiotics

The combined effect for Tyrosol/gentamicin, tyrosol/amikacin and tyrosol/ciprofloxacin was measured by Checkerboard microdilution method (Orhan et al., 2005). The concentration of each drug ranged from 2-fold to 1/32 MIC. One hundred microliter of each antibiotic was combined with 100 µl of tyrosol in 96 well microtitre plates. The plates were inoculated with 20 µl of an overnight LB culture giving a final concentration of 5.0×10^5 CFU/ml per well. Plates were incubated for 24 h at 37°C. To evaluate the interaction, the fractional inhibitory concentration index (FICI) was calculated for each combination according to White et al. (1996) and Abdelmegeed and Shaaban (2013).

Measuring the effect of tyrosol on metabolic activity

To evaluate the effect of tyrosol on vitality of *S. aureus* isolates, MTT reduction assay was conducted on both tyrosol treated and non-treated cells at a defined time point (Montoro et al., 2005). First, a suspension of an overnight *S. aureus* culture (100 µl) was dispensed into selected wells of a 96-well plate containing tyrosol (3.5-228.8 mM) and incubated for 24 h at 37°C. Next, 10 µl of MTT solution [5 g/l in phosphate buffered saline (PBS) (10 mM Na_2PO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.0), filter sterilize by bacterial filter (0.22 µm)] was added to each well and the plate was re-incubated for 4 h. Aspiration of the well solution was performed followed by addition of 50 µL of dimethylsulfoxide (DMSO) solution to the wells. The plate was shaken for 5 min. A change in color from yellow to violet was measured with a microtitre plate reader (BioTek ELx800) at 540 nm. All assays were carried out in triplicate.

Biofilm formation

Biofilm was developed in a 96 well microtiter plate (Nunc, New York, NY, USA). *S. aureus* cultures (100 µl) were incubated for 24 h at 37°C in the presence of different concentrations of tyrosol (3.5-14.3 mM) and the procedure was completed according to Eid et al. (2012) and El-Mowafy et al. (2014).

Effect of tyrosol on *S. aureus* virulence factors

The effect of tyrosol on the production of total protease, lipase and lecithinase enzymes by tested isolates was done with formerly prepared culture supernatant. Total protease activity was measured using the modified skim milk assay as previously described (El-Mowafy et al., 2014). The lipase assay was carried out using p-nitrophenyl palmitate as a substrate as previously described (EL-Sokkary et al., 2011).

The lecithinase activity was measured by mixing 100 µl of the filtrate with or without tyrosol and 100 µl of the egg-yolk suspension (egg-yolk tellurite emulsion 20 ml/L; NaCl, 5 g/L). The pH was adjusted to 7.8 in individual wells of polystyrene 96-well plates then the plates were incubated at 37°C for 24 h and absorbance was measured at 600 nm using microplate reader. All experiments were carried out in triplicates.

Statistical analysis

Statistical analysis was done using GraphPad Prism. One way

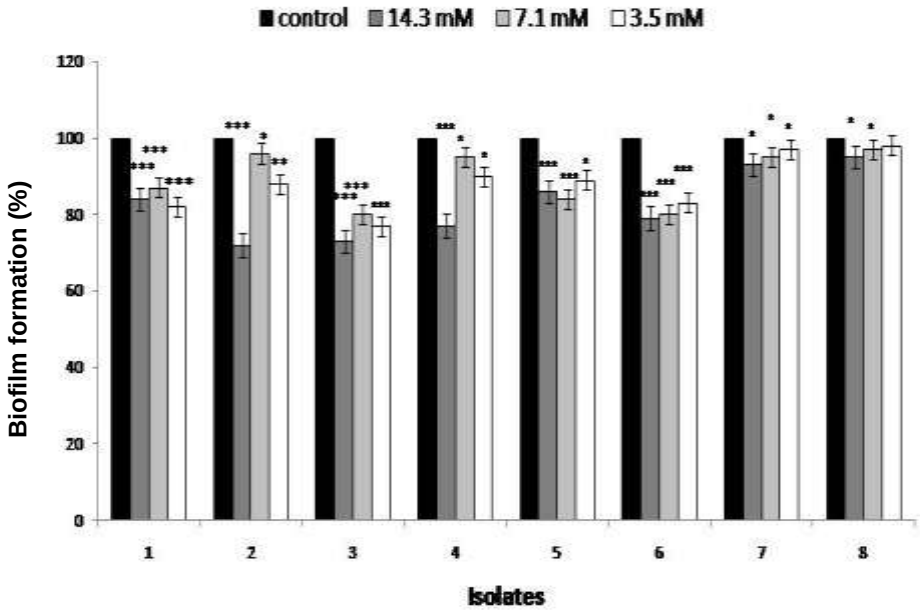

Figure 1. The effect of subinhibitory concentrations (3.5-14.3 mM) of tyrosol on biofilm formation. (***: P≤0.0001, **: P≤0.001, *: P≤0.05).

ANOVA followed by Turky posttest were performed to compare the differences between the control and treated isolates. A P value ≤ 0.05 was considered statistically significant.

RESULTS

MIC determination

The MIC of tyrosol, gentamicin, amikacin and ciprofloxacin was determined by microbroth dilution method. The MIC of tyrosol was found to be 114.4 mM for all the tested isolates. The MIC for gentamicin, amikacin and ciprofloxacin were 62.5-8000, 17.5-70 and 125-500 µg/ml, respectively.

The combined effect of tyrosol and antibiotics

The effect of tyrosol/gentamicin combination was found to be synergistic in 66% of tested isolates (FICI=0.28-0.5). Besides that, tyrosol/amikacin and tyrosol/ciprofloxacin combinations exhibited synergistic effect in two isolates (FICI=0.37). For the other isolates, these three combinations showed an additive effect (FICI=0.51-0.62).

Effect of tyrosol on the metabolic activity

MTT, an oxidation-reduction indicator was added to suspensions in the presence of tyrosol. The vitality assay indicated that incubation of cells with 114.4 and 228.8 mM of tyrosol (1x and 2x MIC) resulted in no change in color from yellow to violet, showing that these concentrations prevented the oxidation-reduction reactions in the suspensions. Tyrosol (28.6- 57.2 mM) partially inhibited oxidation-reduction reactions. Lower concentrations (3.5 - 14.3 mM) had no effect on the viability of tested isolates. Therefore, these subinhibitory concentrations were used for studying the effect of tyrosol on the production of virulence factors.

Biofilm formation

The effect of 3.5 - 14.3 mM of tyrosol on biofilm formation was estimated. All the tested concentrations reduced the formed biofilm of the isolates significantly except for one isolate in which (3.5 mM) tyrosol reduced the formed biofilm in non-significant manner (Figure 1). The maximum decrease (30%) in biofilm formation was attained using 3.5 mM of tyrosol among isolates 2, 3 and 4.

Effect of tyrosol on virulence factors

Sub-inhibitory concentrations (3.5 - 14.3 mM) of tyrosol were found to significantly decrease protease and lipase enzyme production in MRSA isolates 3, 5 and 7. Tyrosol (14.3 mM) significantly decreased protease and lipase release with P<0.05 by 35-40% (Figure 2) and 25-40% (Figure 3), respectively. On the other hand, lecithinase enzyme production was increased significantly in all the

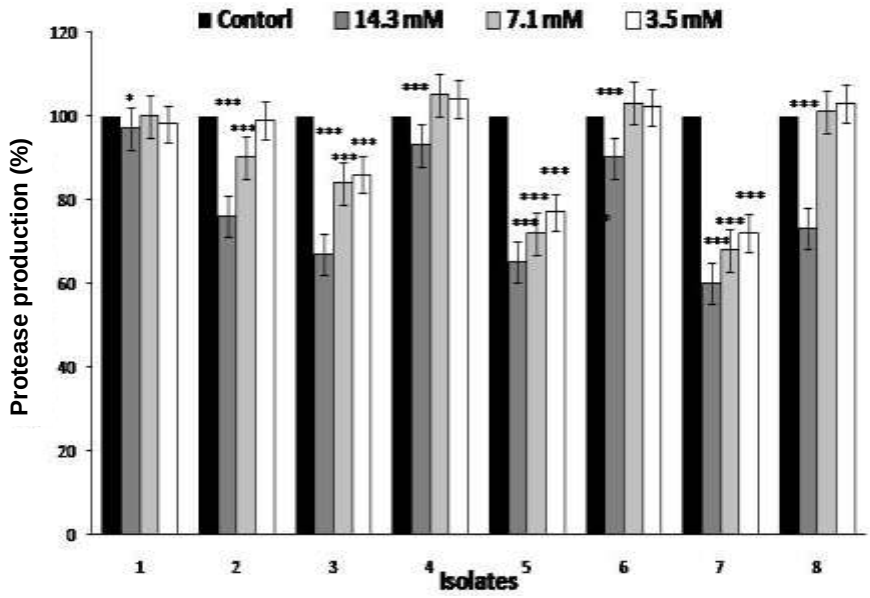

Figure 2. The effect of subinhibitory concentrations (3.5-14.3 mM) of tyrosol on protease enzyme production. (***; P≤0.0001, **; P≤0.001, *; P≤0.05)

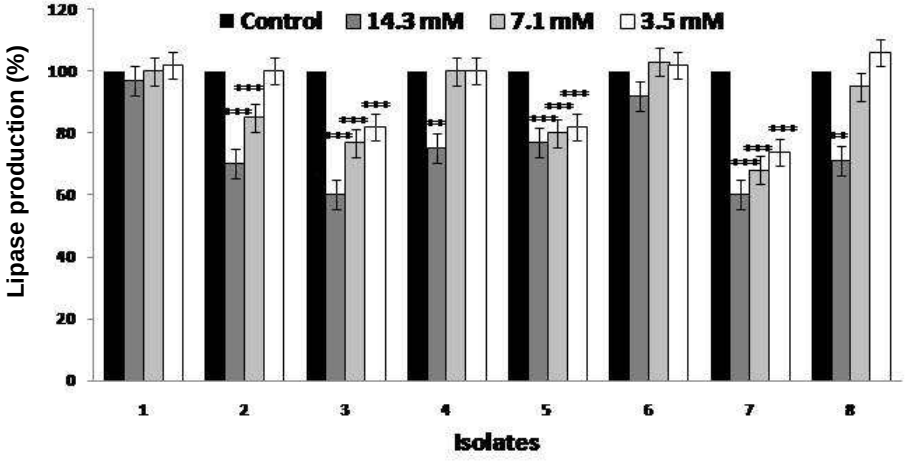

Figure 3. The effect of subinhibitory concentrations (3.5-14.3 mM) of tyrosol on lipase enzyme production. (***: P≤0.0001, **: P≤0.001, *: P≤0.05).

tested isolates except for one isolate in which 3.5 mM of tyrosol led to non-significant increase (Figure 4).

DISCUSSION

There is mounting indication in the literature for the prominence of polymicrobial infections in which organisms interact with each other synergistically or inhibiting the effect of their pathogenesis and patient health (Harriott and Noverr, 2009). *C. albicans* cohabits with various human microbiota in multiple sites which give rise to mixed species biofilm (Shirtliff et al., 2009; Peleg et al., 2010; Ovchinnikova et al., 2012). In poly- microbial blood stream infections, *C. albicans* was co-isolated with *S. aureus* in 11% of cases (Klotz et al., 2007).

Besides that, *C. albicans* and *S. aureus* can form complex polymicrobial biofilms on various mucosal surfaces in which *S. aureus* is associated with hyphal cells (Peters et al., 2010). The effect of *C. albians* itself on *S. aureus* has been extensively investigated previously (Harriott and Noverr, 2009; Ovchinnikova et

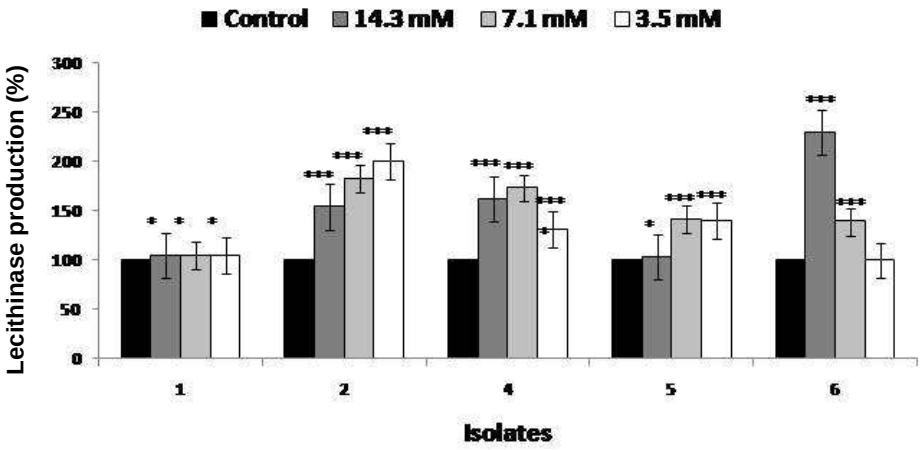

Figure 4. The effect of subinhibitory concentrations (3.5-14.3 mM) of tyrosol on lecithinase enzyme production. (***: P≤0.0001, **: P≤0.001, *: P≤0.05).

al., 2012; Lin et al., 2013; Schlecht et al., 2015). The effect of farnesol, *C. albicans* quorum sensing compound, was studied on *S. aureus* in various reports (Unnanuntana et al., 2009; Kaneko et al., 2011; Cerca et al., 2013). Till now, no available investigation was done to evaluate the effect of the other *C. albicans* quorum sensing compound (tyrosol) on the sensitivity of *S. aureus* to antibiotics and virulence production.

In this study, the authors were interested in determining how tyrosol might affect the antibiotic sensitivity, biofilm and some virulence factors production (protease, lipase and lecithinase) by Egyptian clinical isolates of *S. aureus*.

Tyrosol exhibits antibacterial effect against *S. aureus*. In contrast to these results, Papadopoulou et al. (2005) showed that tyrosol is least likely to exhibit any anti-microbial activity on *S. aureus*, *E. coli* and *C. albicans.* Being a phenolic compound may account for this antibacterial activity by having membrane-active properties which induce progressive leakage of intracellular constituents, including K^+ (Lambert and Hammond, 1973). Aside from being a quorum sensing compound, tyrosol is one of the simple phenols found in olive oil providing benefits to health. Medina et al. (2006) reported that hydroxytyrosol and tyrosol are olive oil phenolic compounds having bactericidal activity against the tested foodborne pathogens including *S. aureus.*

The ability of tyrosol to sensitize the tested isolates to antibiotics (gentamicin, amikacin and ciprofloxacin) was assessed. It enhanced the activity of the three antibiotics against *S. aureus* isolates. About 66.7% of tested isolates exhibited sensitivity towards the tyrosol/gentamicin combination. For amikacin and ciprofloxacin, tyrosol exhibited a synergistic effect in two out of eight isolates and showed additive effect in the other isolates. The synergistic effect shown by tyrosol/ antibiotic com-binations may be explained by the possible membrane permealizing action of tyrosol which increases the sensitivity of *S. aureus* to antibiotics. A similar finding was reported by Jabra-Rizk et al. (2006) who showed that farnesol increases the sensitivity of tested mecithillin sensitive *S. aureus* isolates to gentamicin and this increase was not shown in MRSA isolates. Unnanuntana et al. (2009) have demonstrated that farnesol/gentamicin combination has no effect on the sensitivity of *S. aureus* to gentamicin. Concerning farnesol, it has variable activities against different organisms in combination with different antimicrobials. For example, a study performed by Abdel-Rhman et al. (2015) showed that neither farnesol nor tyrosol at their subinhibitory concentrations had any effect on the activity of antibiotics on *Pseudomonas aeruginosa*.

Bacterial biofilm is a microbial derived sessile community, where cells are embedded in extracellular polymeric matrix. Cells in biofilm show an altered phenotype in growth, gene expression and protein production. Biofilm formation requires first adherence of organisms to surfaces, followed by proliferation and finally biofilm formation (Jabra-Rizk et al., 2006; Unnanuntana et al., 2009). In the current study, subinhibitory concentrations of tyrosol reduced biofilm formation significantly. This result is consistent with that of Arias et al. (2016) who demonstrated that tyrosol reduces single and mixed biofilms formed by *C. albicans* and *Streptococcus mutans*. Also, Jabra-Rizk et al. (2006) found that farnesol has the ability to inhibit biofilm formation by *S. aureus*.

Moreover, the effect of tyrosol on the production of some virulence factors of *S. aureus* was studied. Tyrosol subinhibitory concentrations (3.5-14.3 mM) reduced protease enzyme production in 100, 50 and 37.5% of the tested isolates, respectively. This result coincides with that of Abdel-Rhman et al. (2015) who reported a similar result against *P. aeruginosa*.

Another virulence factor of *S. aureus* is lipase that

contributes to skin colonization through hydrolyzation of human sebum. Moreover, the effect of tyrosol on lipase production was investigated. Tyrosol (14.3 mM) caused 25-40% reduction of lipase production in six isolates, 7.1 mM resulted in 20-30% reduction in four isolates and 3.5 mM resulted in about 20% reduction in only three isolates. These results are consistent with what reported by Kuroda et al. (2007) who found that subinhibitory MIC of farnesol inhibited lipase activity in *S. aureus*. On the contrary, lecithinase was significantly increased by the tested concentrations of tyrosol. None of the previous studies have investigated the effect of either tyrosol or farnesol on lecithinase production. So, this result requires further study to explain this increase by studying its effect on *agr* (accessory gene regulator) quorum-sensing system of *S. aureus* which controls the expression of many secreted virulence factors.

Conclusion

The present study results demonstrate the beneficial effects of tyrosol in relation to antimicrobial susceptibility and virulence factors production of *S. aureus*. This sheds more light on the therapeutic use of tyrosol against *S. aureus* infections. However, more studies are required to reveal its effect on quorum sensing system that controls virulence gene expression.

Conflict of Interests

The authors have not declared any conflict of interest.

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Full Length Research Paper

Effect of non-steroidal anti-inflammatory drugs and dexamethazone on the biofilm formation and expression of some adhesion-related genes of *Candida albicans* and *Staphylococcus aureus*

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Candida albicans* and *Staphylococcus aureus* can cause many diseases which are considered in many cases life threatening infections. Their biofilm formation on the surface of medical devices is difficult to be treated. Our study evaluates the effect of some anti-inflammatory drugs on the growth, biofilm formation and the expression of some adhesion-related genes. Antimicrobial activity of the tested drugs was determined by microbroth dilution method, their effect on biofilm formation was determined by crystal violet assay method, the mechanism of action was assessed by scanning electron microscope and ethidium bromide uptake assay. Their effects on the expression of *icaA, ALS1* and *HWP1* genes were determined by RT-PCR. Diclofenac Sodium had the highest antimicrobial activity followed by Meloxicam. Ethidium bromide uptake increased in the presence of diclofenac sodium in both *S. aureus* and *C. albicans* strains. All tested drugs showed significant inhibitory effect on both biofilm formation and the preformed biofilm. NSAIDs, dexamethazone and ketoconazole down-regulated *C. albicans* tested genes except for ketoprofen that up-regulated *HWP1* gene. NSAIDs and levofloxacin down-regulated the expression *icaA* gene but dexamethazone showed no effect on *icaA* gene expression. Although, dexamethazone had no antimicrobial activity, it had good anti-biofilm activity against *S. aureus* and *C. albicans.

Key words: *Candida albicans, Staphylococcus aureus, biofilm, HWP1, ALS1, icaA, Dexamethazone, NSAIDS.*

INTRODUCTION

Candida spp. can cause opportunistic diseases especially in patients who are immune compromised, aged, receiving prolonged antibacterial and aggressive cancer chemotherapy or undergoing invasive surgical procedures and organ transplantation (Pfaller and Diekma, 2007). *C. albicans* readily forms biofilms on a wide variety of polymers used to make indwelling medical devices, such as dental materials, stents, prostheses,

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implants, endotracheal tubes, pacemakers, and catheters (Douglas, 2003).

S. aureus is found to be common in polymicrobial biofilm infections especially with *C. albicans.* It is estimated that 27% of nosocomial *C. albicans* bloodstream infections are polymicrobial, with *Staphylococcus aureus* (Klotz and Chasin, 2007). Interestingly, the combined effect of *C. albicans* and *S. aureus* results in synergism and increased mortality in mice (Viale and Stefani, 2006).

Different studies have already described changes in gene expression levels during biofilm development (O'Gara, 2007). Adherence of microorganisms to an implanted device is a crucial step in the development of biofilm and biomaterial-entered infection (Seo et al., 2008). Adhesion of *C. albicans* to host cells depends on many adherence molecules such as the agglutinin-like sequence *(ALS)* family, hyphal wall protein *(HWP)* and cell wall glycoproteins. Also, the intercellular adhesin *(ICA)* operon is a virulence factor identified in staphylococci related to biofilm production. As *S. aureus* formation of biofilms requires the synthesis of polymeric N-acetylglucosamine and the enzymes responsible for its synthesis are encoded by the *ica* operon (Gotz, 2002).

The biofilm-associated microorganisms are refractory to both antimicrobial agents and the host immune response. The resistance of biofilm forming microorganisms to antimicrobials represents a major challenge facing most of therapeutic and prophylactic strategies. Re-evaluation of the existing drugs of known mechanisms of action may result in identifying new mechanisms and targets that can affect bacterial metabolism (Golia et al., 2011).

Non-steroidal anti-inflammatory drugs (NSAIDs) are usually prescribed with or without antibiotics for patients had fever or pain due to an infectious condition, musculoskeletal condition (e.g. rheumatoid arthritis, gout), painful condition (e.g. metastatic bone pain, trauma, migraine headache), or the prophylaxis of ischemic heart disease. NSAIDs inhibit the cyclooxygenase enzymes COX-1 and COX-2, which are involved in the biosynthesis of mammalian prostaglandins (Pina-vaz et al., 2000).

Glucocorticoids (GC) have both immunosuppressive and anti-inflammatory actions so that they are used in the treatment of various immune-mediated inflammatory disorders, such as asthma, psoriasis, rheumatism, and multiple sclerosis (MS). GC also inhibits some transcriptt-ion factors, such as nuclear factor kappa B and AP-1 (Auphan et al., 1995). As a result, the transcription of several cytokines that are involved in inflammation is decreased and the production of lipid mediators such as prostaglandins is inhibited and the expression of ICAM-1 and E-selectin on endothelial cells is reduced (Scheinman et al., 1995).

It was found that *C. albicans* can produce prostaglandins but their role in fungal biology is not yet known (Alem and Douglas, 2005). Prostaglandins can act as regulators of *C. albicans* eicosanoids pathway which have a vital role in controlling both morphogenesis and biofilm formation. Also the release of drugs that can target fungal prostaglandins pathways may combat fungal colonization and infection (Erb-Downward and Noverr, 2007). Glucocorticoids are used in many preparations in combination with antifungals and antibacterials (topical skin preparation, eye drops and ear drops). So, we thought to test the *in-vitro* activity of NSAIDs and dexamethasone on the growth, adherence, biofilm formation and the expression of some genes affect adhesion of *C. albicans* and *S. aureus* to know if the tested drugs have any additional effect on the microbial infection or not.

MATERIALS AND METHODS

Microbial strains

Two *C. albicans* and two *S. aureus* clinical strains obtained from the Department Of Microbiology And Immunology, Faculty of Pharmacy, Minia University were used in this study. *S. aureus* (ATCC 6538) and *C. albicans* ATCC 10231 were obtained from MIRCIN culture collection of the Faculty of Agriculture, Ain Shams University, Egypt.

Drugs

Stock solutions of Diclofenac sodium (150.4 mM), Ketoprofen (78.4 mM), Meloxicam (112 mM), Piroxicam (120 mM), Dexamethasone (4 mg/ml), Levofloxacin (122.8mM) and Ketoconazole (74.24) (Sigma) were prepared in Dimethyl sulfoxide (DMSO).

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC) by microbroth dilution method

According to the guidelines of Clinical and Laboratory Standards Institute (CLSI, 2008), the microbial suspensions of *S. aureus* and *C. albicans* cultures were made in sterile normal saline (0.89% NaCl wt/vol; Himedia, India) and the turbidity were adjusted to 0.5 McFarland standards (equivalent to 1.5×10^8 colony forming units (CFU) /ml). Two-fold serial dilutions of tested drugs were prepared in Mueller Hinton Broth (MHB; Difco Laboratories) and dispensed in 96-well microtiter plates (Tarson, India). The microbial suspensions was added to each well of the plate resulting in the final inoculum of 5×10^5 CFU/ml. Levofloxacin was used as standard antibacterial agent while ketoconazole was used as standard antifungal agent. DMSO was used as negative control (show no antibacterial or antifungal activity). The plates were incubated at 37°C for 18 h and were visually read for the absence or presence of turbidity. The minimum concentration of the tested agents showing no turbidity was recorded as MIC. The MBC was determined by spreading 100 µl on tryptic soy agar (Himedia, India) from the wells showing no visible growth. The plates were incubated at 37°C for overnight. Concentrations showed no growth on the surface of TSA was considered as MBC (CLSI, 2008).

Scanning electron microscopy (SEM)

To study the effect of the tested agents on the cell morphology of *S.*

Table 1. Primers used for RT-PCR experiments.

Primer		Sequence (5'–3')
icaA	Probe	TGGATGTTGGTTCCAGAAACATTGGGAG
	Forward	TGAACCGCTTGCCATGTG
	Reverse	CACGCGTTGCTTCCAAAGA
ALS1	Forward	CCTATCTGACTAAGACTGCACC
	Reverse	ACAGTTGGATTTGGCAGTGGA
HWP1	Forward	CTCCAGCCACTGAAACACCA
	Reverse	GGTGGAATGGAAGCTTCTGGA
β-actin gene (ACT1)	Forward	CGTTGTTCCAATTTACGCTGGT
	Reverse	TGTTCGAAATCCAAAGCAACG

aureus and *C. albicans*, SEM was used.

After one hour contact with the MIC concentrations of the tested drugs, the cells were prefixed in 2% glutaraldehyde for one hour at 4°C. Post–fixation was done using a 2% osmium tetroxyd solution. After each fixation, the cells were washed twice with PBS. The samples were gold covered by cathodic spraying (Edwards S 150 B). Finally, the samples were examined as described with the scanning electron microscope (Stereoscann 360, Cambridge) (Benyahya et al., 1992).

Biofilm susceptibility assay

The biofilms of *S. aureus strains (*ATCC 6538 and two clinical strains) and *C. albicans strains* (ATCC 10231 and 2 clinical strains) were prepared in 96-well polystyrene microtiter plates (Tarson, India), using a method of Wei et al. (2006) with a few modifications. The microbial suspensions were prepared from the overnight grown culture and the turbidity of the suspension was adjusted to 0.7 OD_{610} (1 × 10^9 CFU/ml). Two fold serial dilutions for NSAIDs, dexamethasone, levofloxacin and Ketoconazole were prepared in tryptone soya broth (Difco laboratories) supplemented with 0.5% glucose. Forty microliters of fresh TSB with 0.5% glucose was added to each well, followed by the addition of 60 µl of above microbial suspension. Then, different dilutions of the tested products were added and the plates were incubated for 18 h at 37°C. The plates were washed by phosphate buffer saline (PBS) and biofilms were fixed with methanol for 15 to 30 min, stained with Crystal Violet (Sigma, USA). Biofilm formation was quantified by the addition of 95% ethanol to the crystal violet stained wells and recording the absorbance at 595 nm using a microplate reader (Multiskan spectrum, Finland) (Wei et al., 2006).

The effect of the tested drugs also examined on preformed biofilms. The biofilms were prepared by inoculating the suspension of *S. aureus* and *C. albicans* strains into the wells of a polystyrene microtiter plate as mentioned above. After incubation at 37°C for 18 h, the culture supernatant from each well was decanted and planktonic cells were removed by washing the wells with PBS (pH 7.2). Two fold serial dilutions of NSAIDs, dexamethasone, levofloxacin and ketoconazole were prepared in trypticase soy broth (TSB) and 200 µl of each dilution was added to the biofilm in the wells. The plate was further incubated at 37°C for 18 h. The biofilm was fixed, stained and quantified as described above (Wei et al., 2006).

Ethidium bromide accumulation assay

The action of NSAIDs, dexamethasone, levofloxacin and ketoconazole on cell membrane permeability of *S. aureus* ATCC 29213 and *C. albicans* ATCC 10231 cells were evaluated by the method as described by Cox et al. (2000). The microbial cells were grown overnight in Muller Hinton broth (MHB), resuspended in 50-mmol/l sodium phosphate buffer. The turbidity of the suspension was adjusted to 0.7 OD_{610} (1 × 10^9 CFU/ml) and 1 ml of it was added to flask containing 19 ml buffer and the MIC of the tested levofloxacin, ketoconazole, NSAIDs and dexamethasone at 4 mg/ml. Following 60 and 120 min incubation at room temperature, 200 µl aliquots were transferred into tubes containing 3.8 ml phosphate buffer. These tubes were stored on ice and 20 µl of staining solution, consisting of ethidium bromide (Sigma) (10 µg/ml) dissolved in milliQ water. Then, the fluorescence was measured using A Perkin Elmer 45 luminescence spectrometer connected to the FLWINLAB software (United Kingdom) (Cox et al., 2000).

Germ Tube Formation

C. albicans ATCC 10231 colonies were suspended in sterile saline and adjusted to density of 0.5 Mcfarland. Human serum was added to 1 ml of cell suspensions of C. albicans ATCC 10231. Drugs were added to the suspension at MIC concentration and saline was added to the control tube. The cell suspensions were incubated with gentle shaking at 37°C for 2 h and were examined for the presence of germ tubes by using a light microscope. Photomicrographs of colonies and invasive growth were taken with a DMRXA microscope (Leica, Germany) (Liu et al., 1994).

RT-PCR analysis of *C. albicans* adhesion-related genes

Total RNA was extracted from C. albicans biofilms using QIAGEN RNeasy Mini kit (cat no.74524, Germany) according to the guidelines of manufacturer. RNA concentrations and RNA purity were determined using a spectrophotometer (GeneQuart 1300, Germany). An equal amount of RNA was subjected to cDNA synthesis using the cDNA Reverse Transcriptase reagent kit (Applied Biosystems, cat no. 1311190, USA). Real-time PCR primers were designed for the target genes *ALS1*, *HWP1* using Real Time Equipment (Strategene MXP 3000, Germany). The β-actin gene (*ACT1*) was used as an endogenous reference gene. The sequences of the primers are shown in Table 1. QuantiTect®SYBR® Green PCR Kit (Applied Biosystems, cat no. 204141, USA) was used. All PCR reaction mixtures contained: 10 µl QuantiTect®SYBR® Green PCR Master Mix (2X), 2 µl first strand cDNA, 0.5 µl each primer, 0.4 µl ROX Reference Dye (50X) and RNase-H2O to the final volume of 20 µl. The program for amplification was 95°C for 15min as an initial denaturation step to

Table 2. Antimicrobial activity of the tested drugs against *S. aureus* and *C. albicans* strains.

Drug	MIC[a] *(MBC)*[b]			MIC[a] *(MBC)*[b]		
	***S. aureus* ATCC 6538**	***S. aureus* 1**	***S. aureus* 2**	***C. albicans* ATCC 10231**	***C. albicans* 1**	***C. albicans* 2**
Piroxicam	15 (30)	7.5 (7.5)	15 (30)	7.5 (15)	15 (30)	15 (30)
Meloxicam	3.5 (7)	3.5 (7)	1.25 (3.5)	7 (14)	3.5 (7)	3.5 (7)
Diclofenac	4.7 (9.4)	4.7 (18.8)	2.35 (4.7)	0.16 (0.32)	0.08 (0.32)	0.32 (0.32)
Ketoprofen	9.8 (19.6)	4.9 (9.8)	4.9 (9.8)	19.6 (19.6)	19.6 (39.2)	9.8 (39.2)
Dexamethasone	NA	NA	NA	NA	NA	NA
Levofloxacin	0.03 (0.06)	0.015 (0.03)	0.015 (0.03)	NT	NT	NT
Ketoconazole	NT	NT	NT	2.35 (4.7)	1.17 (2.35)	0.58 (1.17)

[a]Minimum inhibitory concentration in mM; [b]Minimum bactericidal concentration in mM; NA, no activity; NT, not tested.

activate the HotStartTaq® DNA polymerase, followed by 40 cycles of PCR consisting of 94°C for 15 s, 60°C for 30 s and 72°C for 30 s. Negative controls (water as template) were included in each run. After amplification, a melting curve was analyzed to confirm the specificity of the primers. Expression of each investigated gene was normalized to the housekeeping *ACT1* gene and analyzed using comparative Ct method (ΔΔCt). Expression of *ALS1* and *HWP1*, genes from cells grown under drug treatment was indicated. Each experimental condition was performed in duplicate and each experiment was repeated twice (Ding et al., 2014).

RT-PCR analysis of *S. aureus* adhesion-related gene

TaqMan®Fast Universal quantitative Real-time PCR for *icaA* gene. Taqman primers and probe are summarized in Table 1. Probe was labeled with the reporter dye 6-carboxyfluorescein (6'-FAM) at the 5'end and with the quencher dye 6-carboxy-tetramethylrodamine (TAMRA) at the 3'end. TaqMan®Fast Universal PCR Kit (Applied Biosystems, cat no.4351891, USA) was used. Thermal cycling conditions were as follows: 10 min at 95°C followed by 45 repeats of 15 s at 95°C, and 1 min at 60°C. Data collection was performed during each annealing phase. Each experimental condition was performed in duplicate and each experiment was repeated twice (Paniagua-Contreras et al., 2012).

Statistical analysis

Data were described as mean ± SD. All statistical analyses were performed by statistical analysis computer software package SPSS 17.0 (SPSS Inc., IL, USA). Student's t-test or one-way ANOVA were used to compare the biofilm formation, planktonic growth, and the gene expression of *C. albicans* and *S. aureus* strains in the presence or absence of drugs. Results with a p-value less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

The attachment of microbial cells to surfaces and accumulation of these cells in forming multilayered cell clusters are key steps in any infection (Ji et al., 1995). In this regard, adhesion formation is considered as one of the major virulence factors in *C. albicans* and *S. aureus*.

C. albicans is the fourth leading cause of bloodstream infections and the third most commonly isolated organism from intravascular catheters and is associated with the highest incidence of mortality (Crump and Colligan, 2000). *C. albicans* and *S. aureus* readily form biofilms on a wide variety of polymers used to make indwelling medical devices (Kojic and Darouiche, 2004). There is some evidence to suggest that a large proportion of device-related *C. albicans* infections involve biofilms (Dominic et al., 2007). In addition, severe catheter-related *S. aureus* infections have been reported in many studies to be important causes of morbidity, mortality, and a source of concern in the primary and emergency care context over the past decade (Parker and Doebbeling, 2012).

In recent years, due to the increased resistance of many bacteria to the commonly used antimicrobial agents, attention has shifted to drugs belonging to different pharmacological classes for possible antimicrobial activity. Many studies showed that NSAIDs have antibacterial activity, antifungal activity and decrease adherence and biofilm formation by bacteria and fungi (Ashraf et al., 2015; Mohsen et al., 2015). Our results showed that Meloxicam and Diclofenac sodium had the highest activity against the tested strains. Diclofenac sodium exhibited higher anti-candidal activity than that showed by Ketoconazole while Levofloxacin had the highest antibacterial activity against *S. aureus* followed by Meloxicam and Diclofenac sodium. Dexamethasone showed no activity against the tested strains (Table 2). Also, we tested the synergistic activity among NSAIDs, dexamethasone and the tested standard antimicrobials; it was found that the tested NSAIDs increased the activity of the tested antimicrobials but dexamethasone showed no increase in their activity (data not shown). The combined effect of antifungal or antibacterial agents and COX inhibitors in Candida infections was reported in many studies (Pina-vaz et al., 2000; Abdelmegeed and Shaaban, 2013).

The increase in the uptake of ethidium bromide is an evidence to the effect of the tested agents on the membrane integrity in comparison to the unexposed cells

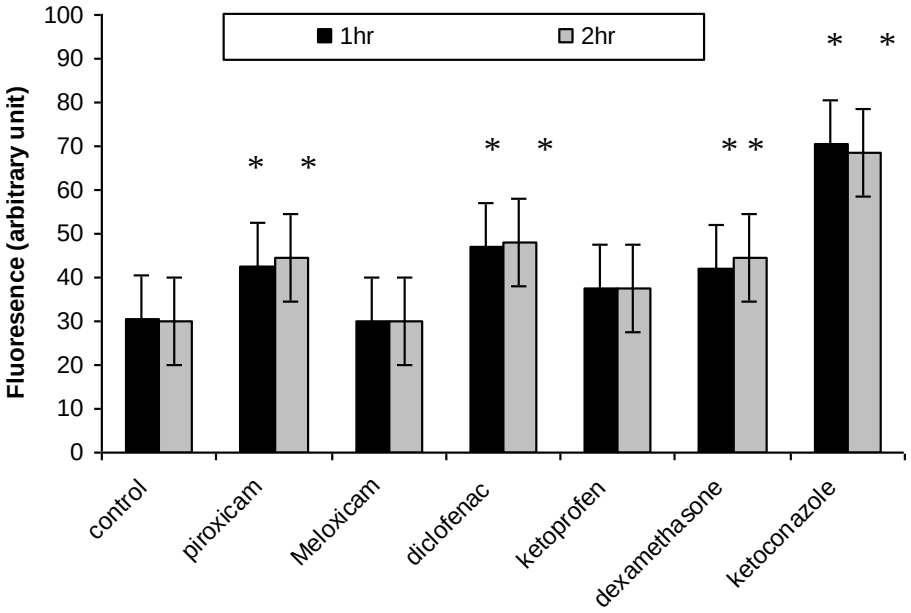


Figure 1. Uptake of ethidium bromide in a cell of *C. albicans* ATCC 10231. Control group (untreated cells). Cells treated with Ketoconazole, Dexamethazone, Diclofenac Sodium and Piroxicam significantly increased the fluorescence compared with untreated control ($P < 0.05$). Data represent the mean and standard deviations (±SD) of two different experiments performed in triplicate. * $P < 0.05$ (Student's t test).

($P<0.05$). It was found that Meloxicam and Levofloxacin showed no increase in the uptake of ethidium bromide by *S. aureus*. For *C. albicans*, Meloxicam showed no effect while Ketoconazole showed the highest disruptive effect on the membrane integrity followed by Diclofenac sodium. As observed, Diclofenac sod had the highest effect on the membrane integrity of both *S. aureus* and *C. albicans* which may explain its antimicrobial effect on both organisms (Figures 1 and 2). Also, the antifungal activity of Diclofenac sodium against *C. albicans* may be due to that their log P (4.75) which are close to the log P of Ketoconazole (4.35). Niazi et al. (2010) explained that the antifungal activity of two Schiff bases may be due to their logP (3.77 and 3.74) which are close to Ketoconazole (4.35). Many studies agreed with our results, as they reported that the antimicrobial activity of NSAIDs may be due to their inhibition of bacterial DNA synthesis or impairment of membrane activity (Niazi et al., 2010; Dutta et al., 2004).

By testing the effect of NSAIDs, Dexamethazone, Levofloxacin and Ketoconazole on the ability of the tested strains to form biofilm, it was found that meloxicam showed the highest ability to inhibit biofilm formation by both *S. aureus* and *C. albicans* strains followed by Diclofenac sodium, Dexamethasone, Levofloxacin (in case of *S. aureus*) or Ketoconazole (in case of *C. albicans*), Ketoprofen and Piroxicam (Figure 3). On the other hand, Diclofenac sodium exhibited the highest disruptive effect on the preformed biofilm formed by the tested strains followed by Meloxicam, Dexamethasone, Levofloxacin (in case of *S. aureus*) or Ketoconazole (in case of *C. albicans*), Ketoprofen and Piroxicam (Figure 4).

Our study showed that Dexamethasone affect the membrane permeability of the *C. albicans* while showed slight effect of *S. aureus* permeability in comparison to controls and its effect on biofilm formation and preformed biofilm are close to that shown by Ketoconazole and Levofloxacin. SEM was used to verify the effect of the tested drugs on the biofilm and the cell morphology of the tested strains. SEM graphs showed that the tested anti-inflammatory drugs caused morphological deformities (*S. aureus* and *C. albicans*) and pore formation on the surface of *C. albicans.* Also, the graphs showed that Dexamethazone caused swelling and pore formation to *C. albicans* cells (Figure 5). But Dexamethazone did not affect the cell morphology of *S. aureus*. The effect of NSAIDs on C. *albicans* and *S. aureus* cell morphology were also detected (Ashraf et al., 2015; Mohsen et al., 2015).

The ability of germ tube formation by *C*. *albicans* in the presence of tested drugs was determined. Diclofenac, Ketoconazole, Meloxicam (at MIC) and Dexamethazone (1 mg/ml) were found to inhibit germ tube formation but Ketoprofen and Piroxicam decreased the amount of germ tubes (Figure 6). Abdelmegeed and Shaaban (2013) reported that cultures treated with some NSAIDs exhibited a dose-dependent inhibition of germ tube

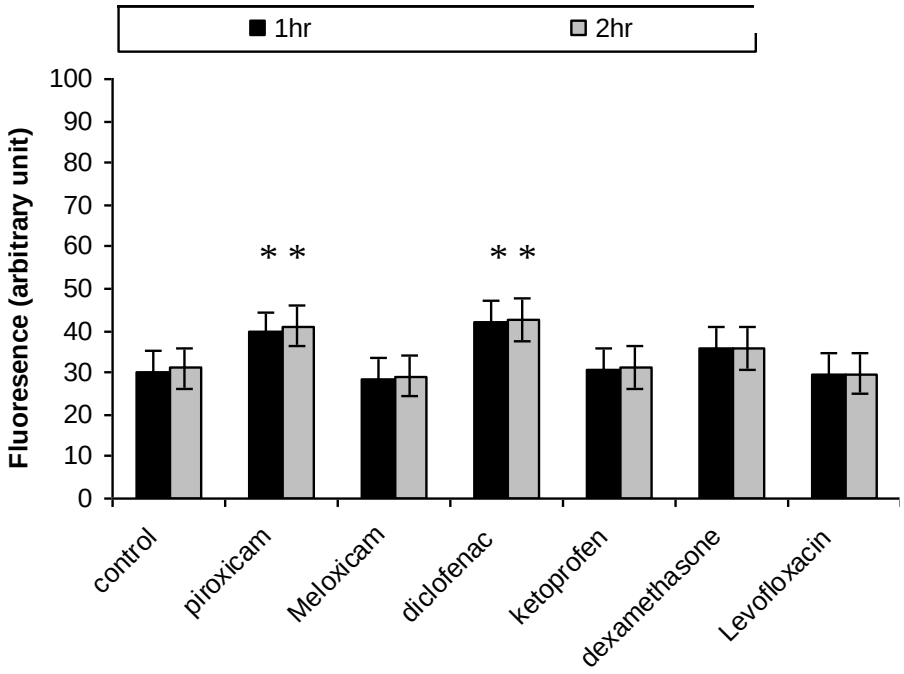


Figure 2. Uptake of ethidium bromide by *S. aureus* ATCC 6538. Control group (untreated cells). Cells treated with Diclofenac Sodium and Piroxicam significantly increased the fluorescence compared with untreated control ($P < 0.05$). Data represent the mean and standard deviations (±SD) of two different experiments performed in triplicate. * $P < 0.05$ (Student's t test).

formation following 3 h incubation at 37°C. They reported significant reduction in germ tube formation under treatment with 1 and 10 mM anti-inflammatory drugs for 3 h especially with Ketorolac, Piroxicam and Diclofenac. Brooks and Day (1991) reported the antigerm tube effect of Diclofenac sodium suggesting that the antigerm tube effect observed *in vitro* might also be relevant in vivo (Brooks and Day, 1991).

Several studies demonstrated that local steroid treatment, even without antibiotics, can substantially reduce *S. aureus* colonization and the therapeutic effect of early combined therapy was superior to that in the steroid-only group; however, at the end of the treatment period, there was no significant difference between the 2 treatment groups in clinical scoring (Gong et al., 2006). According to our results, Dexamethazone had no antibacterial activity, slight effect on permeability of *S. aureus* cell membrane but had significant inhibitory effect on biofilm formation. So, we suggest that its effect may be due to low log P (partition coefficient) (1.83) which increase the wettability of tissue culture plate (TCP) surface and decrease the hydrophobic interaction between *S. aureus* surface and the plastic surface of TCP. Also, its low log P decreased its permeability through the hydrophobic membrane of *S. aureus* (Wang et al., 2012). Its effect on *C. albicans* may be due to its effect on prostaglandin production. The role of prostaglandin E2 (PGE2) and the fungal-produced prostaglandin EX (PGEx) in *C. albicans* in biofilm development and fungal pathogenesis has been demonstrated (Erb-Downward and Noverr, 2007). Moreover it has been investigated by Alem and Douglas (2005) that prostaglandin synthesis by both planktonic and biofilm cells was sensitive to the cyclooxygenase inhibitors aspirin, Diclofenac, and Etodolac. Also, they found that NSAIDs that showed more activity on COX2 (Meloxicam, Diclofenac sodium) had the highest ability to decrease germ tube and biofilm formation (Alem and Douglas, 2005). This explain the variation in activity between the tested drugs as meloxicam and Diclofenac sodium which showed higher effects on germ tube and biofilm formation while Piroxicam showed the lowest effect as it has a COX 1: COX 2 activity ratio of 250:1 (Frölich, 1997). Dexamethazone cause the repression of COX2 and prostaglandin E2 release in human pulmonary A549 cells by transcriptional and post transcriptional mechanisms. So, we suggested that the inhibitory effect of Dexamethazone may be due to its effect on COX2 and PGE2 that play an important role in hyphae formation and host cell damage (Mishra et al., 2014).

Due to the observed effect of the tested drugs on biofilm formation and the preformed biofilms, we tried to determine the potential molecular mechanism behind the ability of the tested agents to prevent biofilm formation by both *C. albicans* and *S. aureus*. We have provided evidence that there were variable effects of the tested

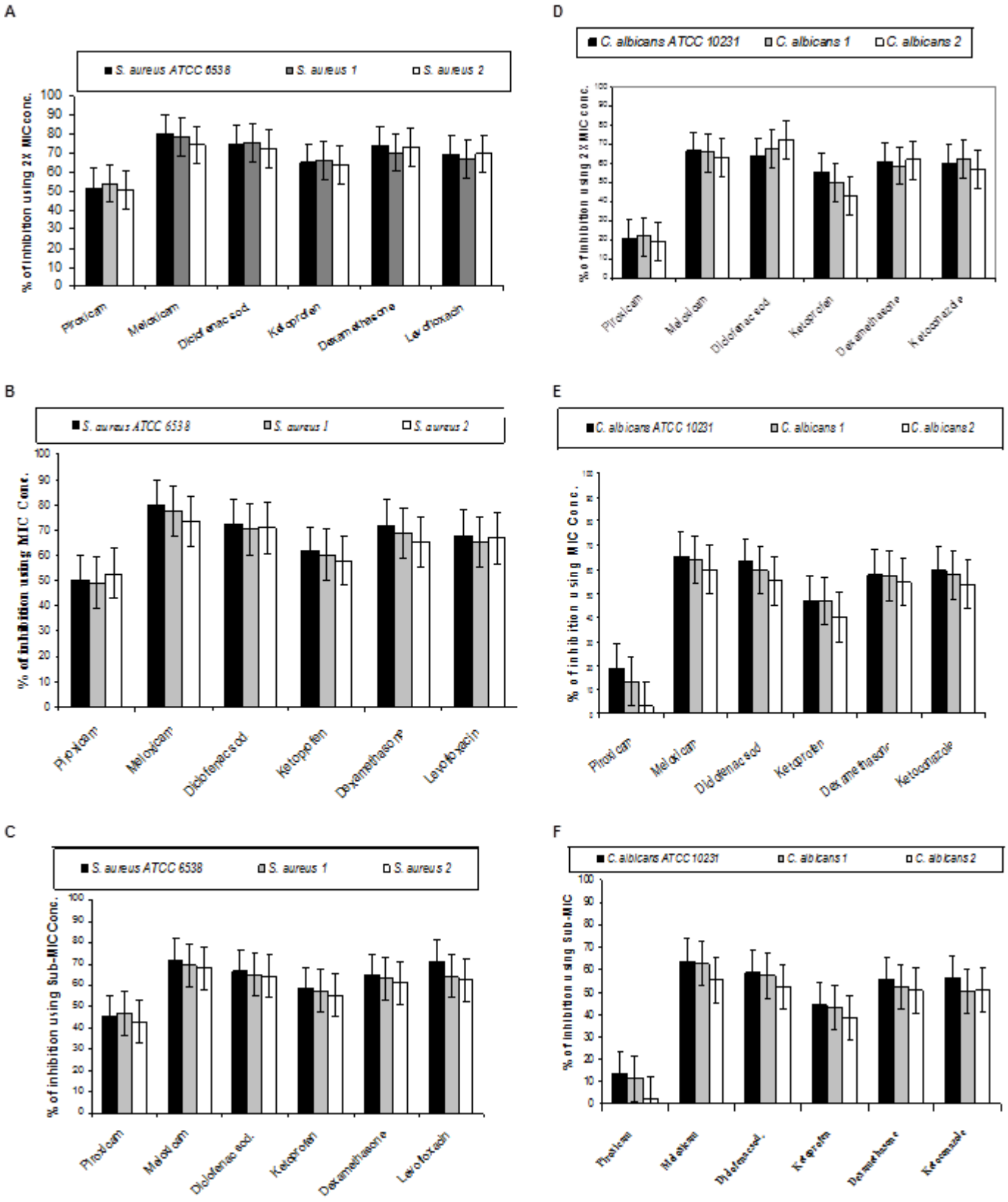


Figure 3. Effect of the tested agents on biofilm formation of both *S. aureus* (A, B & C) and *C. albicans* (D, E & F) strains. The results are expressed as average optical density readings for crystal violet assays compared to growth control. The biofilm of *S. aureus* and *C. albicans* were reduced with all tested agents ($P < 0.01$) while Piroxicam showed low effect on *C. albicans* ($P < 0.05$) compared with those of control. Values are mean (±SD) from four independent determinations (Student's t test).

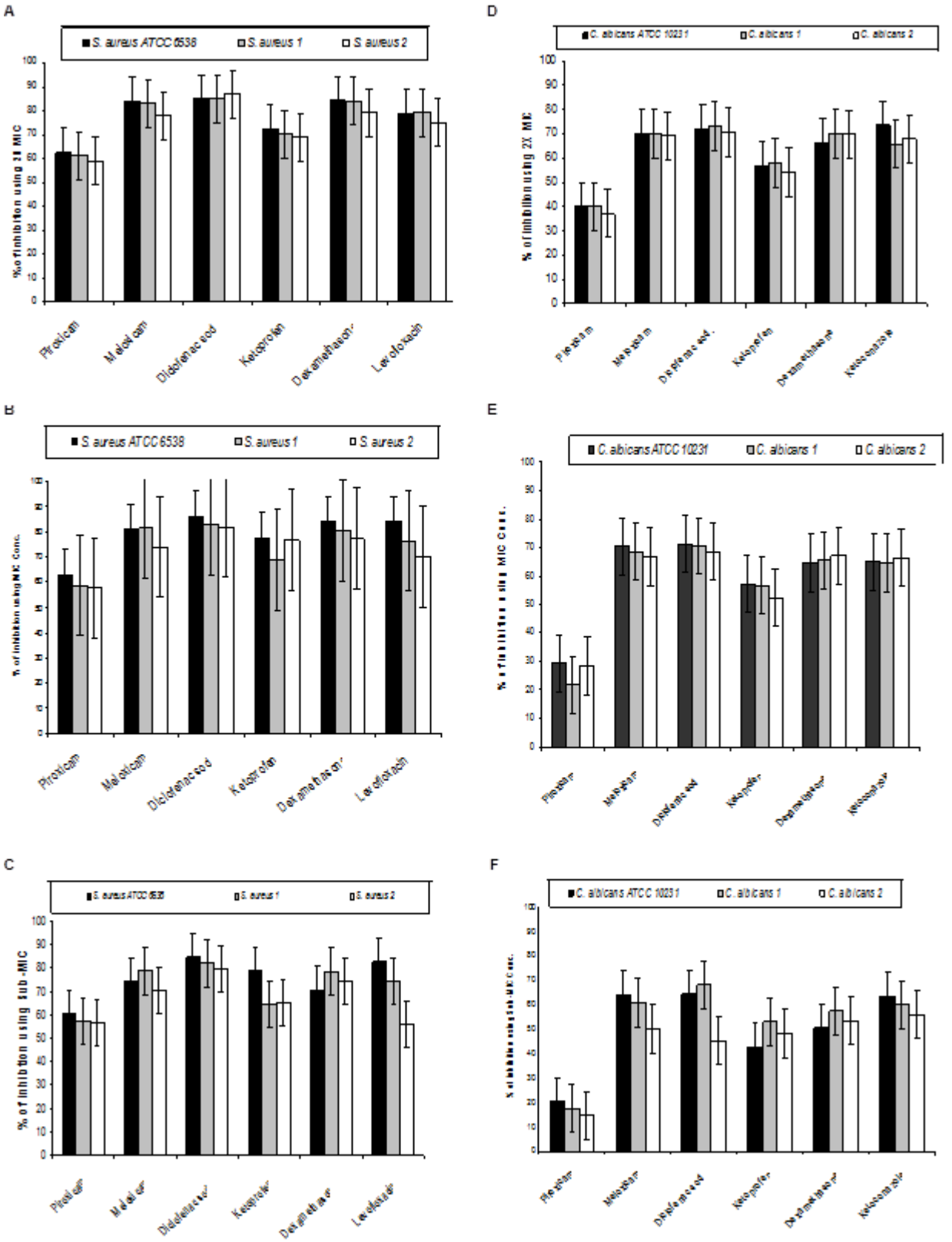
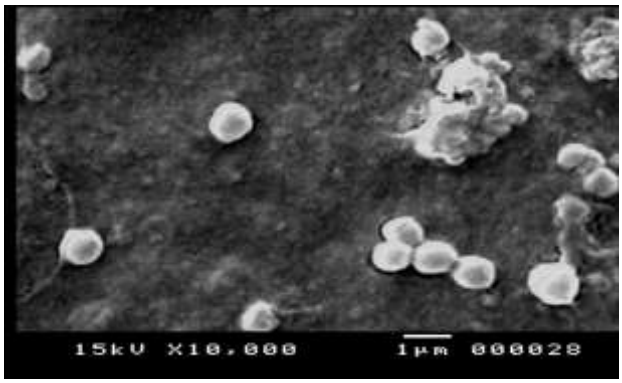
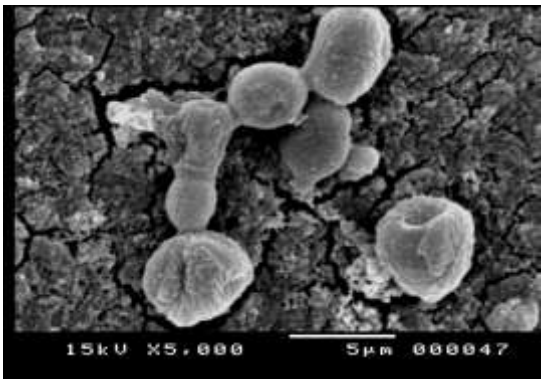


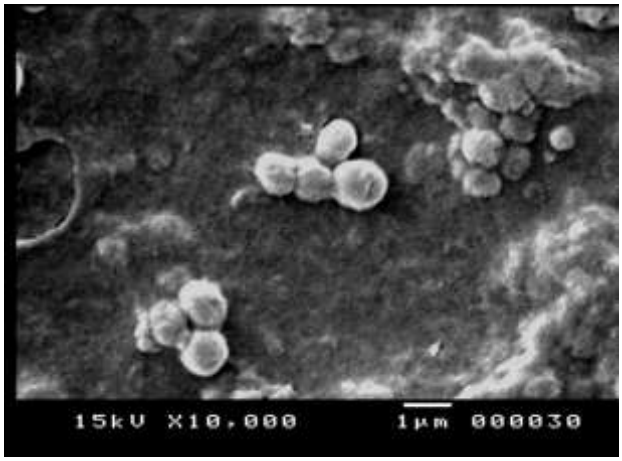
Figure 4. Effect of the tested agents on preformed biofilm of both *S. aureus* (A, B & C) and *C. albicans* (D, E & F) strains. The results are expressed as average optical density readings for crystal violet assays compared to growth control. The biofilm of *S. aureus* and *C. albicans* were reduced with all tested agents ($P < 0.01$) while Piroxicam showed low effect on *C. albicans* ($P < 0.05$) compared with those of control. Values are mean (±SD) from four independent determinations (Student's t test).



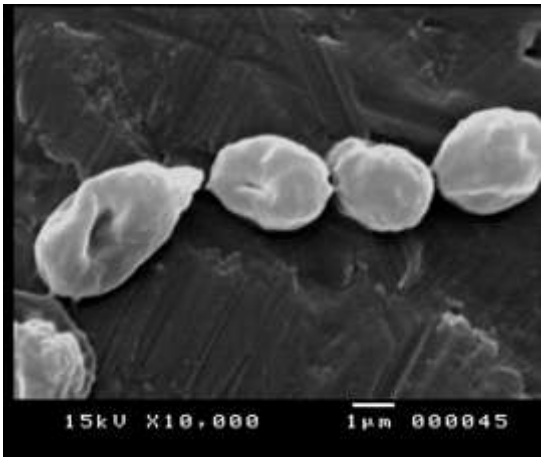
(A) Control *S. aureus*



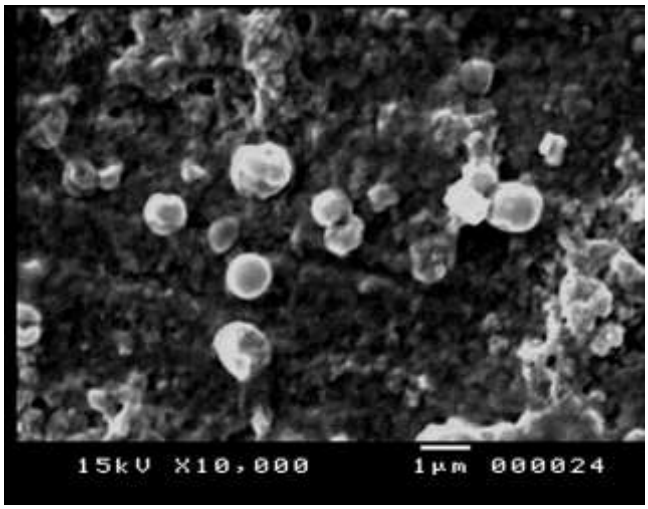
***(D)C. albicans* treated with 10 mM dexamethasone**



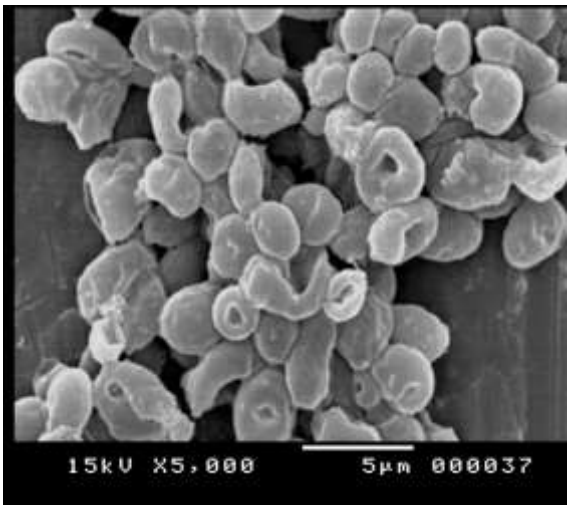
***(B) S. aureus* treated with meloxicam at MIC**



***(E) C. albicans* treated with meloxicam at MIC**



(C) *S. aureus* treated with diclofenac sod. at MIC



***(F) C. albicans* treated with diclofenac sod. at MIC**

Figure 5. Scanning electron micrographs of *S. aureus* untreated (A), treated with Meloxicam (B) and Diclofenac sodium (C) (Swollen with cell wall deformities) and *C. albicans treated with dexamethazone* (D), *Meloxicam* (E) *S. aureus* treated with Diclofenac sodium (F) *C. albicans* treated with diclofenac sodium (cells were swollen, wrinkled and with pores).

drugs on the tested genes in comparison to control (*C. albicans* in the presence of serum). All tested drugs down-regulated the expression of the adhesion-related gene *ALS1* (1.0643 to 2 fold). Dexamethasone was found to down-regulate the expression of *ALS1* by 2 fold while Ketoconazole by 1.197 fold. On other hand, it was found

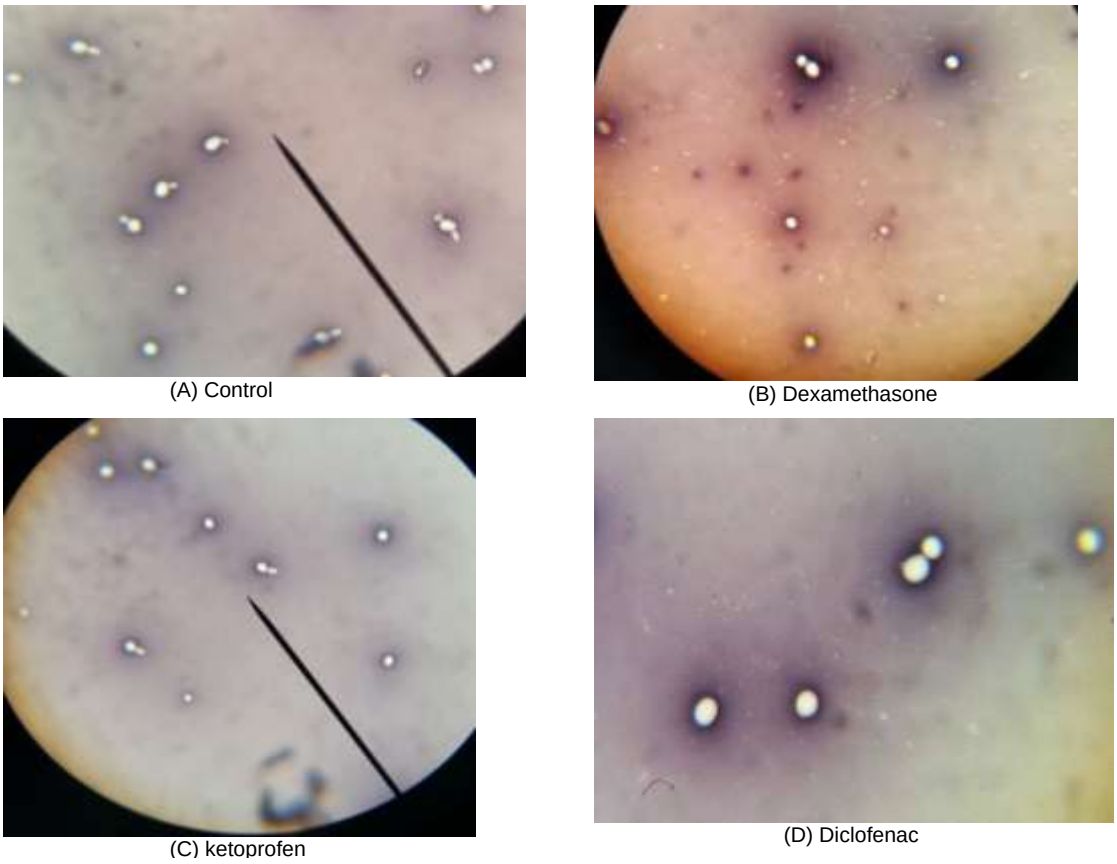


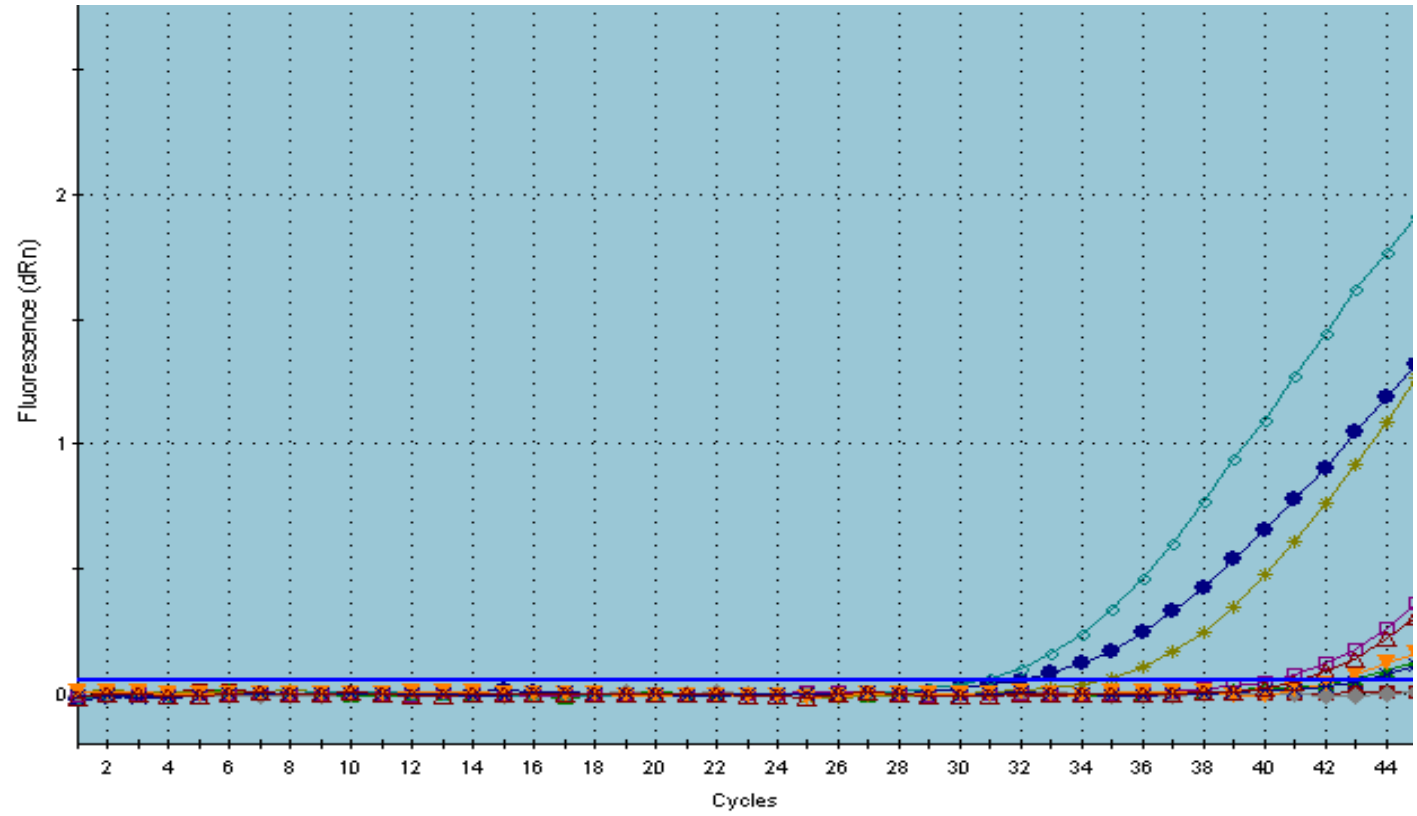
Figure 6. Microscopical examination of yeast-filament transition of *C. albicans* ATCC 10231. Yeast cells with no hypha (B& D) or few hyphal cells (C), compared to (A) control untreated cells.

that Ketoprofen up-regulated the expression of *HWP1* gene by 3.2 fold. Meloxican down-regulated *HWP1* expression by 4.11 fold followed by Ketoconazole (3.89 fold) and Piroxicam (3.81) (Figure 7). The morphological switch from yeast-to-hypha is one of the most important biological features that enable *C. albicans* to colonize, invade, and survive in the host tissues during infection. *C. albicans*, the yeast-to-hypha transition is triggered by various environmental causes, such as serum, N-acetylglucosamine, neutral pH, high temperature, starvation, CO_2, and adherence (Biswas et al., 2007). In responses to a wide variety of stresses, including nutritional and environmental stresses, *C. albicans* initiate morphological changes which are controlled by a complex network of parallel pathways (e.g. MAPK and cAMP-PKA pathways) (Garcia-Rodriguez et al., 2005). Thus, any interference with the expression of genes involved in the MAPK cascades and cAMP-PKA pathway can block filamentation in *C. albicans*. The ALS family gene is related to the growth and morphological change of *C. albicans*. ALS family especially, *ALS1* and *ALS3* are important in the yeast-to-hypha transformation of *C. albicans* (Hoyer, 2001). Another well-characterized *C. albicans* hypha specific gene is the glycosylphosphatidylinositol (GPI)-linked cell surface protein *HWP1*. Functional analyses showed that this protein is required for the adherence of fungal cells to epithelial cells as well as for normal biofilm and hypha formation repression effect of Diclofenac sodium on the regulation of genes controlling *C. albicans* morphogenesis was also reported (Loza et al., 2004; Zhou et al., 2012).

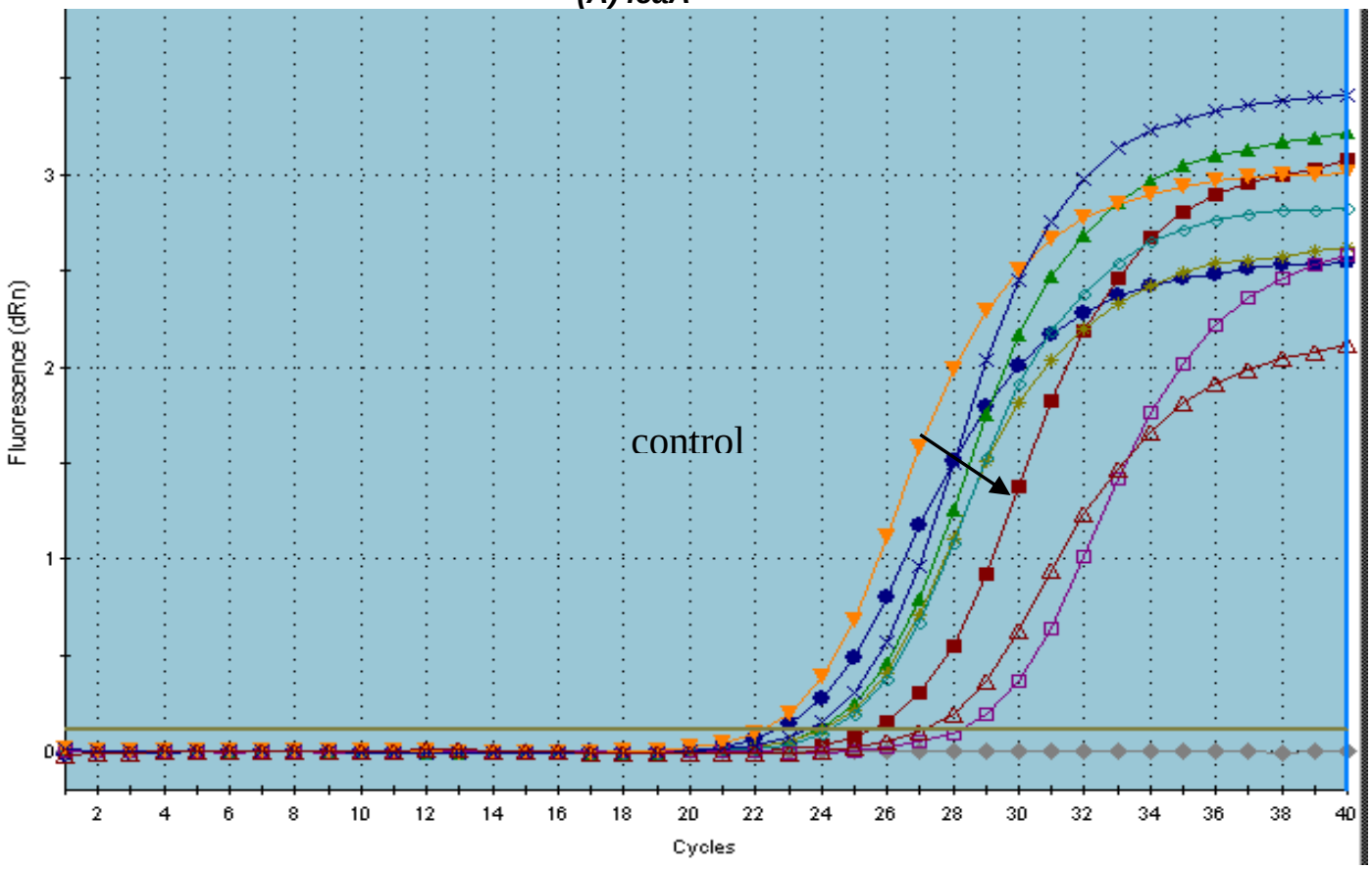
S. aureus biofilm formation requires the production of polymeric N-acetylglucosamine that is controlled by *icaABCD* operon. Meloxicam was found to down-regulate the expression of *icaA* gene by 17.46 fold followed by levofloxacin (15.62 fold) and diclofenac sod. (7.042 fold) but dexamethasone showed no significant effect on its expression (Table 3).

Conclusion

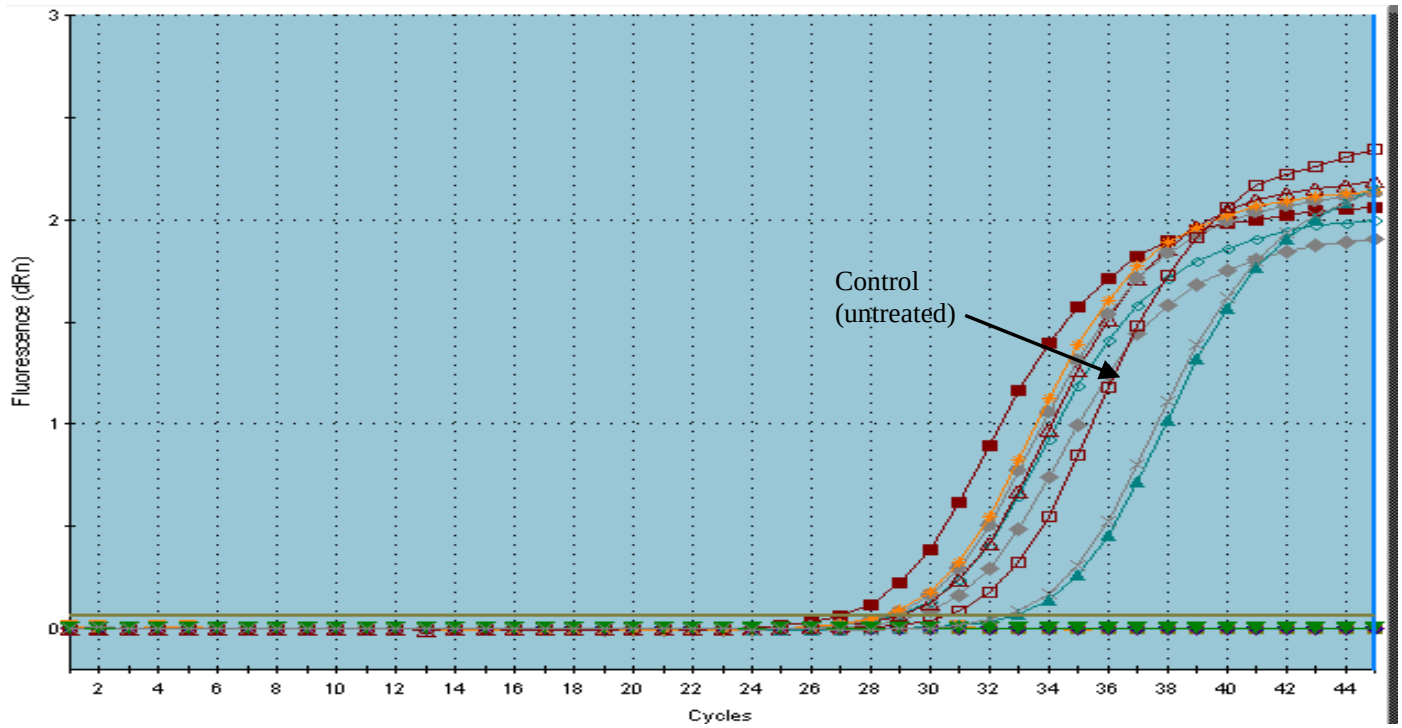
NSAIDs have antibacterial and antifungal activity, can affect membrane permeability, germ tube formation and the expression of some genes controlling adhesion. Dexamethazone showed significant effect on biofilm formation of both *S. aureus* and *C. albicans* which may



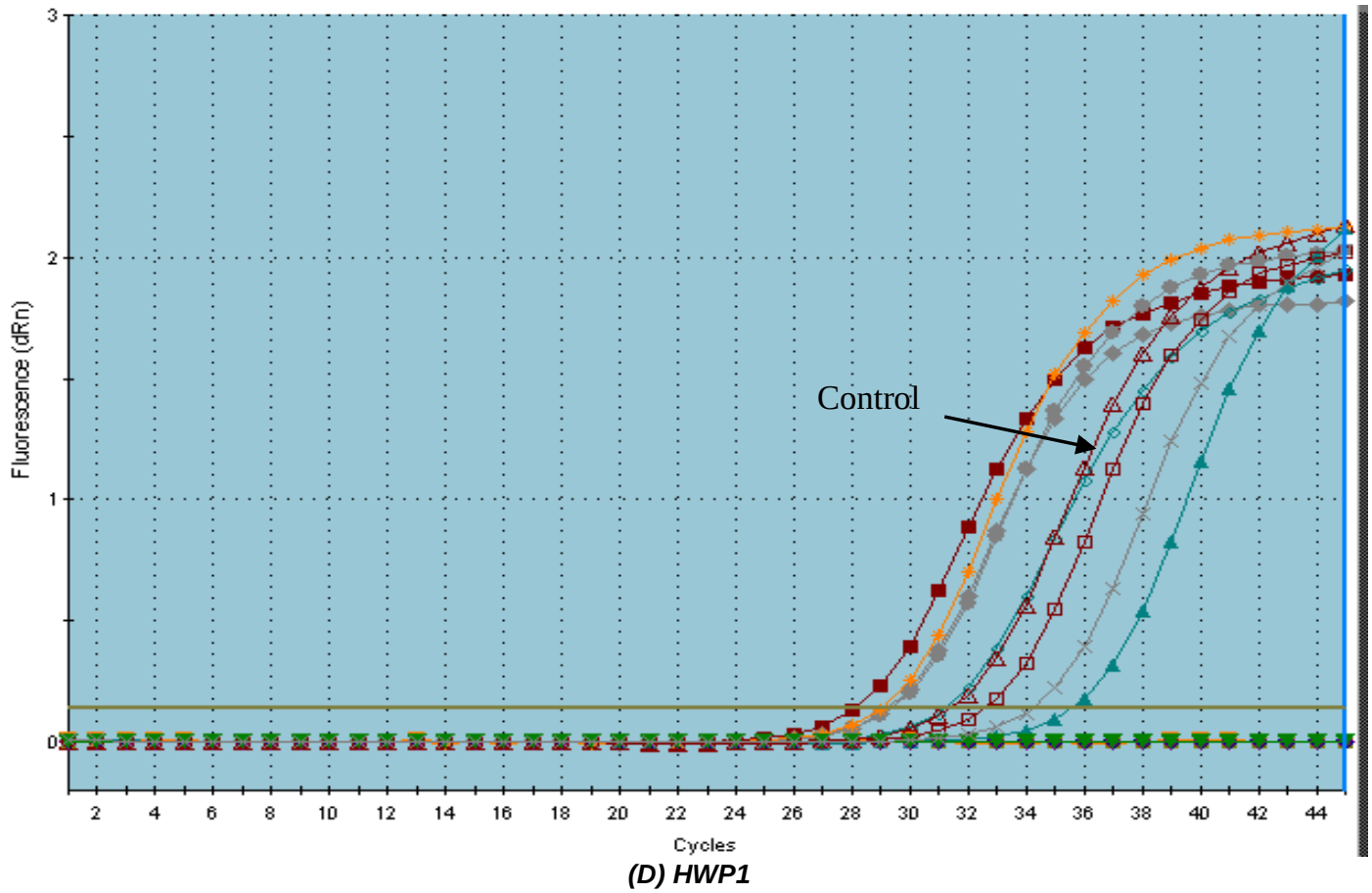
(A) icaA



(B) ACT1



(C) ALS1



(D) HWP1

Figure 7. Effect of NSAIDs, dexamethasone and levofloxacin on the expression of *icaA* gene in *S. aureus* (A) and the effect of NSAIDs, dexamethasone and ketoconazole on the expression of *ALS1* (C) and *HWP1 (D)* genes in *C. albicans*. *ACT1* housekeeping gene (B).

Table 3. Effect of the tested drugs on the expression of adhesion-related genes.

Tested drugs	*C. albicans* (*ALS1*)		*C. albicans* (*HWP1*)		*S. aureus* (*icaA*)	
	Down-regulated (fold)	Up-regulated (fold)	Down-regulated (fold)	Up-regulated (fold)	Down-regulated (fold)	Up-regulated (fold)
Ketoconazole	1.197	-	3.89	-	NT	-
Levofloxacin	NT	-	NT	-	15.62	-
Meloxicam	1.624	-	4.11	-	17.46	-
Piroxicam	1.635	-	3.81	-	7.04	-
Diclofenac	1.0643	-	1.5	-	2.89	-
Ketoprofen	1.22	-	-	3.2	0.4	-
Dexamethasone	2	-	2.62	-	-	-

be a result of its Log P and the inhibition of PGEs release and affected gene expression of *ALS1* and *HWP1* of *C. albicans.* These effects of the tested drugs in addition to its anti-inflammatory properties may result in good response to the antimicrobial therapy *in-vivo*.

Conflict of Interests

The authors have not declared any conflict of interests.

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Effect of cholesterol lowering multiplex lactic acid bacteria on lipid metabolism in a hamster model

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This study aimed to investigate the effects of a probiotic complex product including *Pediococcus*, *Lactobacillus*, and *Bifidobacteria* on the lipid metabolism of hamsters fed a high-fat and high-cholesterol diet. Fifty male Syrian hamsters were assigned to five experimental groups: control, high-fat plus high-cholesterol diet (HFC), and HFC supplemented with low-, medium-, and high-dose of probiotic product. The hamsters in the control group were fed an AIN-76 basal diet. A high-cholesterol diet was based on the AIN-76 basal diet, supplemented with 0.5% (w/w) cholesterol, 12% corn oil, and 3% (w/w) lard to adjust the fat content. After a one-week adaptation period, the experimental period started, during which the animals were fed for 10 weeks, and food intake and body weight were recorded periodically. Blood samples were obtained for the analysis of serum total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), and thiobarbituric acid reactive substances (TBARS). The results show that feeding animals with the low, medium, and high doses of probiotic complex product had significantly lowered serum LDL-C, and TBARS levels, as well as LDL-C/HDL-C ratio ($P < 0.05$), while we observed a significant increase in HDL-C levels ($P < 0.05$). These results indicate that the probiotic complex product could reduce obesity, dyslipidemia, and lipid peroxidation.

Key words: Probiotics, cholesterol, LDL-C/HDL-C ratio, thiobarbituric acid reactive substances, hamster.

INTRODUCTION

In 2013, the World Health Organization (WHO) reported that cardiovascular diseases (CVDs) were responsible for 30% of deaths worldwide, and by 2030, CVDs would affect approximately 23.3 million people around the world (WHO, 2013). According to Hjermann et al. (1981), every 1% reduction in serum cholesterol lowers the risk of coronary heart diseases by 2%. Although drug therapies effectively decrease cholesterol levels, they are expensive and have some side effects. For example, myopathy was found to occur in 10% of statin-treated patients

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(Moosmann and Behl, 2004; Eckel, 2010), and gastrointestinal discomfort such as constipation was described in patients consuming bile-salt sequestrants (Davidson et al., 1999). Moreover, cognitive impairment was reported in patients administered with lovastatin or simvastatin (Muldoon et al., 2000, 2004).

Probiotics are defined as live microorganisms which, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO, 2002). Probiotics were reported to have cholesterol-lowering effects *in vitro* or *in vivo,* especially the strains of genera Lactobacillus and Bifidobacterium (Pan et al., 2011; Wang et al., 2012; Huang et al., 2013; Hu et al., 2013; Tsai et al., 2014). In recent years, several mechanisms for cholesterol removal by probiotics have been proposed, such as the cholesterol assimilation into bacterial cell membranes (Kimoto et al., 2002), deconjugation of bile salts by bile-salt hydrolase (BSH) (Ahn et al., 2003; Tsai et al., 2014), and production of short-chain fatty acids (SCFAs) during growth of probiotics (Tabuchi et al., 2004).

However, the mechanism underlying the hypocholesterolemic effect of probiotics might be strain-specific. *Lactobacillus fermentum* normally adheres to epithelial cells in the human gastrointestinal tract and promotes the survival of healthy intestinal microflora (Wickström et al., 2013). Several studies have reported beneficial effects of exo-polysaccharides on host health, including cholesterol-lowering effect. For example, the exo-polysaccharide produced by *L. kefiranofaciens*, kefiran, was reported to reduce serum cholesterol levels as well as suppress the blood pressure increase in SHRSP/Hos rats consuming excessive amounts of cholesterol (Maeda et al., 2004). Pigeon et al. (2002) suggested that cholesterol removal by *L. delbrueckii* and *Streptococcus thermophilus* strains was due to the binding of free bile acids to their cell membranes through extracellular polysaccharides. Oral administration of probiotics was shown to significantly reduce cholesterol levels by as much as 22 to 33% (Pereira and Gibson, 2002), or prevent elevated cholesterol levels in mice that had been fed a fat-enriched diet (Taranto et al., 2000).

Previously, we isolated different probiotic isolates from animal and plant sources to evaluate their bile-salt hydrolase activity *in vitro*. We demonstrated that the BSH activity and bile-acid deconjugation abilities of *Pediococcus acidilactici* NBHK002, *B. adolescentis* NBHK006, *L. rhamnosus* NBHK007, and *L. acidophilus* NBHK008 were higher than those of the other probiotic strains. NBHK002, NBHK006 and NBHK007 reduced apo B secretion by 33, 38 and 39%, respectively, after 24 h of incubation. The product PROBIO S-23 caused a greater decrease in the total concentration of cholesterol, low-density lipoprotein, TG and thiobarbituric acid reactive substances in the serum or livers of hamsters with hypercholesterolemia in the pre-induced high blood lipid animal model (fed a high-fat and high-cholesterol diet ten days before the experimental period) (Tsai et al., 2014). Based on these results, the present study was conducted to determine the effect of PROBIO S-23 on cholesterol-lowering in hamsters fed a high-fat and high-cholesterol diet simultaneously with PROBIO S-23 during the experimental period (non-pre-induced high blood lipid animal model).

MATERIALS AND METHODS

Bacterial strains, culture medium and growth conditions

Each lactic acid bacteria (LAB) stock culture was maintained in 20% glycerol at -80°C. Bacterial cells were propagated twice in lactobacilli Man, Rogosa, Sharpe (MRS) broth (DIFCO, Detroit, Michigan, USA), supplemented with 0.05% L-cysteine and incubated at 37°C for 20 h. The freeze dried powder of a novel multispecies probiotic mixture (PROBIO S-23) including *Lactobacillus rhamnosus* NBHK007 (strain LCR177), *Bifidobacterium adolescentis* NBHK006 (strain BA286), and *Pediococcus acidilactici* NBHK002 (strain PA318), were isolated from pickled vegetables and human feces, respectively.

Animals and experimental groups

This experimental protocol (No. 10105) was approved by the Institutional Animal Care and Use Committee of HungKuang University, Taichung, Taiwan. Fifty 3-week-old male hamsters were purchased from the National Laboratory Animal Center (Taipei, Taiwan). They were housed individually in a controlled environment with 20 ± 2°C temperature, 55 ± 5% humidity, and a 12 h light:dark cycle with the light period from 8 AM to 8 PM. During the first week of the acclimatization period, the animals were fed chow pellets (AIN-76; Jinlong Technology Co. Ltd, Taichung, Taiwan) and water *ad libitum*. They were then randomly divided into one control group and four experimental groups, namely, high-fat and high-cholesterol diet (HFC group), and HFC + low- (78 mg/kg BW/day), HFC + medium-(390 mg/kg BW/day), and HFC + high-dose PROBIO S-23 powder (1950 mg/kg BW/day) groups. Hamsters in the four experimental groups were fed a basal AIN-76 diet supplemented with 12% corn oil, 3% lard and 0.5% cholesterol. Simultaneously, during the experimental period (10 weeks), different doses of LAB were orally administered with a sterile orogastric tube once a day to the animals in the three PROBIO S-23 groups, in addition to the HFC diet. PROBIO S-23 powder with high viable counts of LAB (1×10^9 to 1×10^{10} CFU/mg) was produced by freeze-drying (New Bellus Enterprise Co., Ltd, Tainan, Taiwan). Weights of the animals and food intake were recorded. Serum was collected to measure the concentrations of total cholesterol (TC), triglycerol (TG), low-density lipoprotein (LDL), and high-density lipoprotein (HDL), as well as the lipid peroxidation index (thiobarbituric acid reactive substances, TBARS).

The animals were sacrificed after 10 weeks. Livers of the animals from all experimental groups were removed and fixed in 10% neutral formalin for preservation. After fixation in 10% neutral formalin solution, sections were cut from the livers of animals from all groups and examined for histopathology. The formalin-solution-fixed livers were coarsely repaired and processed via dehydration, clarification, paraffin infusion, and embedding steps to prepare paraffin tissue blocks. The slices were stained with hematoxylin & eosin (H&E) and observed by microscopy.

Statistical analysis

Statistical analyses were performed using the SPSS 17.0 software

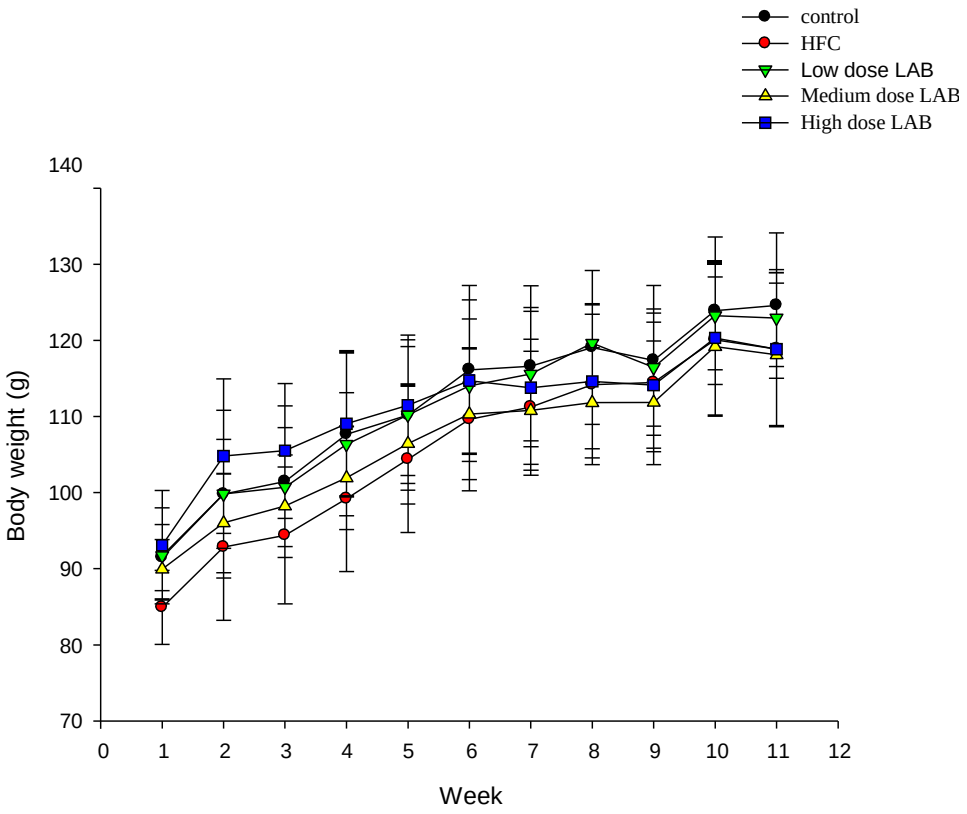

Figure 1. Body weight with high fat plus high cholesterol diet for 10 weeks with different concentrations of probiotics complex product.

(SPSS Inc., Chicago, IL, USA). The data of body weight, TC, TG, LDL-C, HDL-C and TBARS were analyzed by one-way analysis of variance (one-way ANOVA), followed by the Duncan's multiple range test to determine significant differences among groups ($P < 0.05$).

RESULTS AND DISCUSSION

Body weight, the weight percentage gain, and food intake

The mean body weight values of hamsters in the three PROBIO S-23 dosage groups, the HFC group, and the control group are shown in Figure 1. The results of the ANOVA analysis indicated that the final body weight values of the hamsters from the PROBIO S-23 dosage groups and the HFC group were not significantly different from the control group ($P > 0.05$).

On calculating the percentage weight gain during the experiment, we found that the weight percentage gain of the HFC group was approximately 28.5% (Table 1). For hamsters fed high-fat and high-cholesterol diet and simultaneously administered different (low-, medium-, or high-) doses of LAB, the weight percentage gains were 25.4, 23.8, and 21.7%, respectively (Table 1). We noted that, with the increasing dose of LAB the weight percentage gain decreased. Although the HFC diets resulted in overall increase of the body weight in all four experimental groups, we demonstrated that the HFC group without the LAB supplement showed the highest increase in weight percentage gain. Therefore, administering lactic acid bacteria product reduced the body weight gain in the animals that were fed the high-cholesterol and high-fat diet.

The mean food-intake values for the hamsters in different groups during the experimental period are listed in Table 1. No significant differences were observed throughout the study among the three PROBIO S-23 dosage groups, the HFC group and the control group ($P >0.05$). Therefore, we conclude that feeding the mixture of multiple strains of the LAB product did not affect the appetite or digestive function of hamsters. The results obtained here are in concordance with the findings of Huang et al. (2013), who used *L. plantarum* Lp09, an isolate from kefir grains, as supplementation of a high-cholesterol diet. In their study, it was found that there were no significant differences in the total food intake between the groups with or without the supplement; however, they noted lower weight gain and food efficiency in the group administered *L. plantarum* Lp09 than in the high-cholesterol diet group.

Table 1. Body weight gain and food intake of hamsters fed a high-fat plus high-cholesterol diet for 10 weeks, supplemented or not with different concentrations of probiotics complex product, PROBIO S-23.

Group	Body weight (g)		Weight percentage gain (%)	Daily weight gain (g)	Food intake (g /day)
	Initial	Final			
Control	91.48±4.35[a]	124.59±9.56[a]	26.6	0.43±0.06	10.95±2.41[a]
HFC	84.93±4.87[b]	118.85±10.04[a]	28.5	0.44±0.06	10.164±2.35[a]
Low dose LAB	91.72±6.29[a]	122.92±6.33[a]	25.4	0.41±0.08	10.36±2.35[a]
Medium dose LAB	89.93±3.90[a]	118.08±9.43[a]	23.8	0.37±0.05	9.90±2.4[a]
High dose LAB	93.10±7.20[a]	118.84±10.02[a]	21.7	0.33±0.10	9.81±2.50[a]

Data are expressed as means ± SD (n=10). [a,b]Values in the same column with different superscripts mean significant difference ($P < 0.05$). weight percentage gain= (final weight-initial weight) / final weight *100. Daily weight gain = (final weight- initial weight) / days.

Total cholesterol and triglyceride levels in serum

Throughout the 10-week experimental period, the levels of total cholesterol were significantly lower ($P < 0.05$) in the medium- and high dose PROBIO S-23 groups compared with those in the HFC group (Figure 2). Compared with the HFC group, hamsters fed low-, medium-, and high doses of LAB showed total cholesterol levels in serum reduced by 3.30% ($P > 0.05$),10.55% ($P < 0.05$) and 15.13% ($P < 0.05$), respectively, at the end of the trial (Figure 2A).

These results suggest that the higher doses of LAB reduce the serum total cholesterol levels more effectively compared to HFC group. There were no significant differences in triglyceride levels among the three PROBIO S-23 dosage groups and the HFC group (Figure 2B). The base levels of TG at week 0 were significantly different among groups, with the highest level in high-dose LAB group. Probably this is the reason why there were no significant differences in TG levels among the three PROBIO S-23 dosage groups and the HFC group.

High-density lipoprotein and low-density lipoprotein levels in serum, and LDL-C/HDL-C ratio

The levels of high-density lipoprotein cholesterol (HDL-C) were significantly higher ($P <0.05$) in the medium- and high dose PROBIO S-23 groups compared with the HFC group throughout the 10-week experimental period (Figure 3A). Hamsters fed the high dose of LAB showed HDL-C levels increased by 31.1% ($P < 0.05$) compared with the HFC group at the end of the trial (Figure 3A). All three PROBIO S-23 dosage groups showed reduced LDL-C levels and LDL-C/HDL-C ratio at 6, 8, and 10 weeks compared with the HFC group (Figure 3B and C).

Compared with the HFC group, hamsters fed low, medium, and high doses of LAB showed LDL-C levels (LDL-C/HDL-C ratio) in serum reduced by 10.14% (7.64%) ($P < 0.05$), 34.29% (42.64%) ($P < 0.05$) and 42.89% (56.44%) ($P < 0.05$), respectively, at the end of the experimental period (Figure 3B and C). The high-dose PROBIO S-23 group showed the greatest reduction in LDL-C levels at 6, 8 and 10 weeks (by 27.62, 32.79, and 42.89%, respectively) compared with the HFC group (Figure 3B).

The present animal studies indicated not only a significant increase in the concentration of HDL-C but also a significant decrease in the concentrations of LDL-C in the groups administered the PROBIO S-23 product in comparison with the HFC group. The results related to HDL-C and LDL-C are in concordance with previous studies (Abd EI-Gawad et al., 2005; Klein et al., 2008), which demonstrated that animals fed a cholesterol- and fat-richdiet showed reduced total cholesterol levels, LDL-C and increased the HDL-C fraction when supplemented with a daily intake of LAB or yogurt. Some studies have shown that animals fed cholesterol and fat showed a reduction in total cholesterol levels and LDL-C, but HDL-C concentrations did not increase significantly (Jones et al., 2012; Wang et al., 2009; Pan et al., 2010; Huang et al., 2013), while other studies did not observe cholesterol lowering effect following lactic acid bacteria consumption (de Roos, 1999; St-Onge et al., 2002). These conflicting results may be due to the different properties of the LAB strains used such as acid resistance, bile tolerance, or different mechanisms of lowering cholesterol *in vitro* (Akalin et al., 1997; Taranto et al., 1998). Other factors may be involved, e.g., the cholesterol content in diet, LAB ingestion dosage, LAB combinations and their different ratios, animals used, and length of the feeding period (Wang et al., 2009; Starovoitova et al., 2012).

Thiobarbituric acid reactive substances (TBARS) levels in serum

The highest TBARS levels in serum were detected in the HFC group of hamsters from the sixth week to the tenth week of the study. These values were significantly

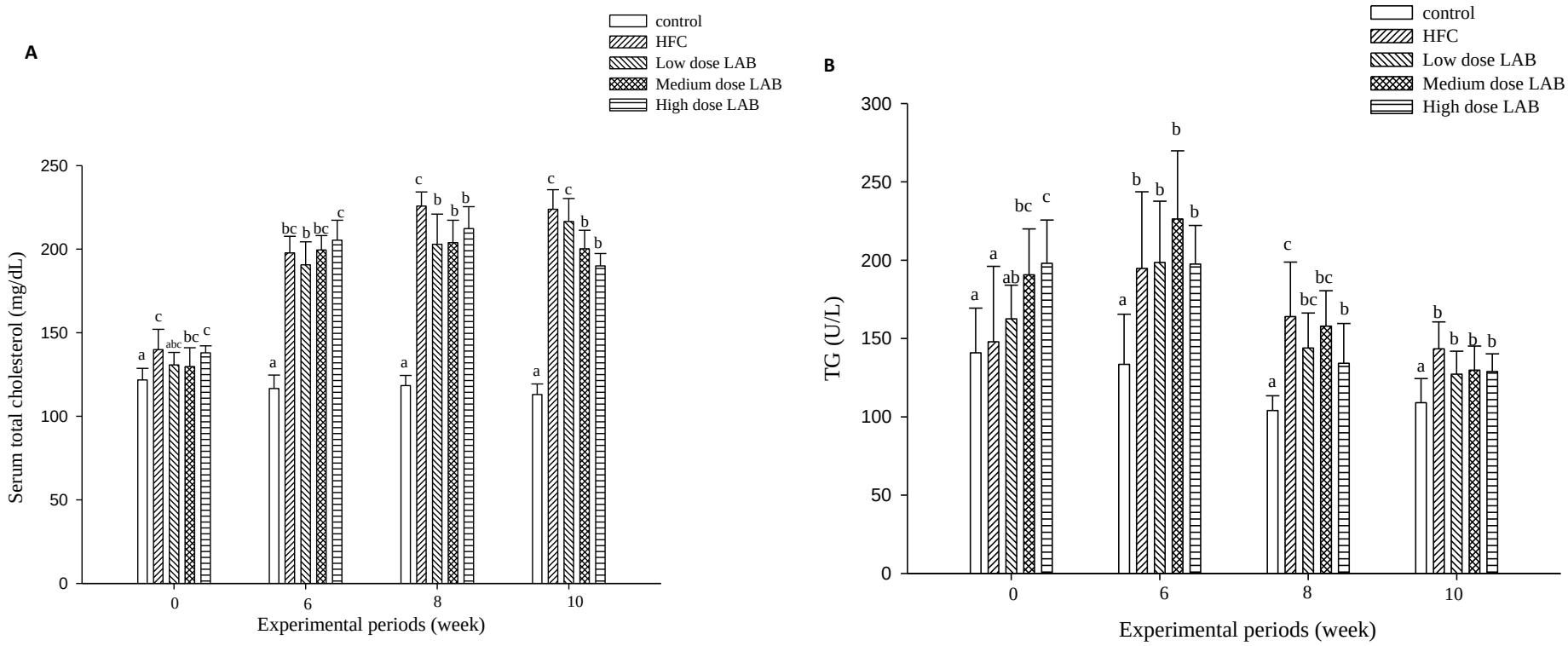

Figure 2. Serum (A) total cholesterol and (B) triglyceride of hamsters fed a high-fat plus high-cholesterol diet and supplemented or not with different concentrations of probiotics complex product, PROBIO S-23, during 10 weeks of the study. Data are expressed as means ± SD (n=8). [a,b c,d]Values in the same column with different superscripts mean significant difference ($P < 0.05$).

different from the control and the three PROBIO S-23 dosage groups ($P < 0.05$). The high-dose PROBIO S-23 group showed the greatest reduction of TBARS levels at 6, 8, and 10 weeks (by 9.86, 43.07 and 49.09%, respectively) compared with the HFC group (Figure 4). Moreover, the malondialdehyde (MDA) levels in hamsters of the high-dose PROBIO S-23 group decreased and were similar to the MDA values in the control group, indicating that the probiotics reduced the effect of lipid peroxidation in serum in the high-fat and high-cholesterol diet.

Being a biomarker of lipid peroxidation, the plasma MDA level is considered a marker of the oxidative stress caused by cholesterol (Nagao et al., 2005). Holvoet et al. (1995) measured plasma MDA-LDL in humans and suggested that an increase in plasma MDA-LDL can be used as a marker of unstable atherosclerotic cardiovascular disease and that blood MDA-LDL is an independent factor not correlated with LDL cholesterol. Previously, MDA-LDL was measured indirectly by using TBARS, and many reports described that catechins prevent serum TBARS increase.

Malondialdehyde is the principal and the most studied product of polyunsaturated fatty acid peroxidation. This aldehyde is a highly toxic molecule and should be considered as more than just a marker of lipid peroxidation. Its interaction with DNA and proteins has often been referred to as potentially mutagenic and atherogenic (Del Rio et al., 2005). Most assays to determine MDA are based on its derivatization with thiobarbituric acid

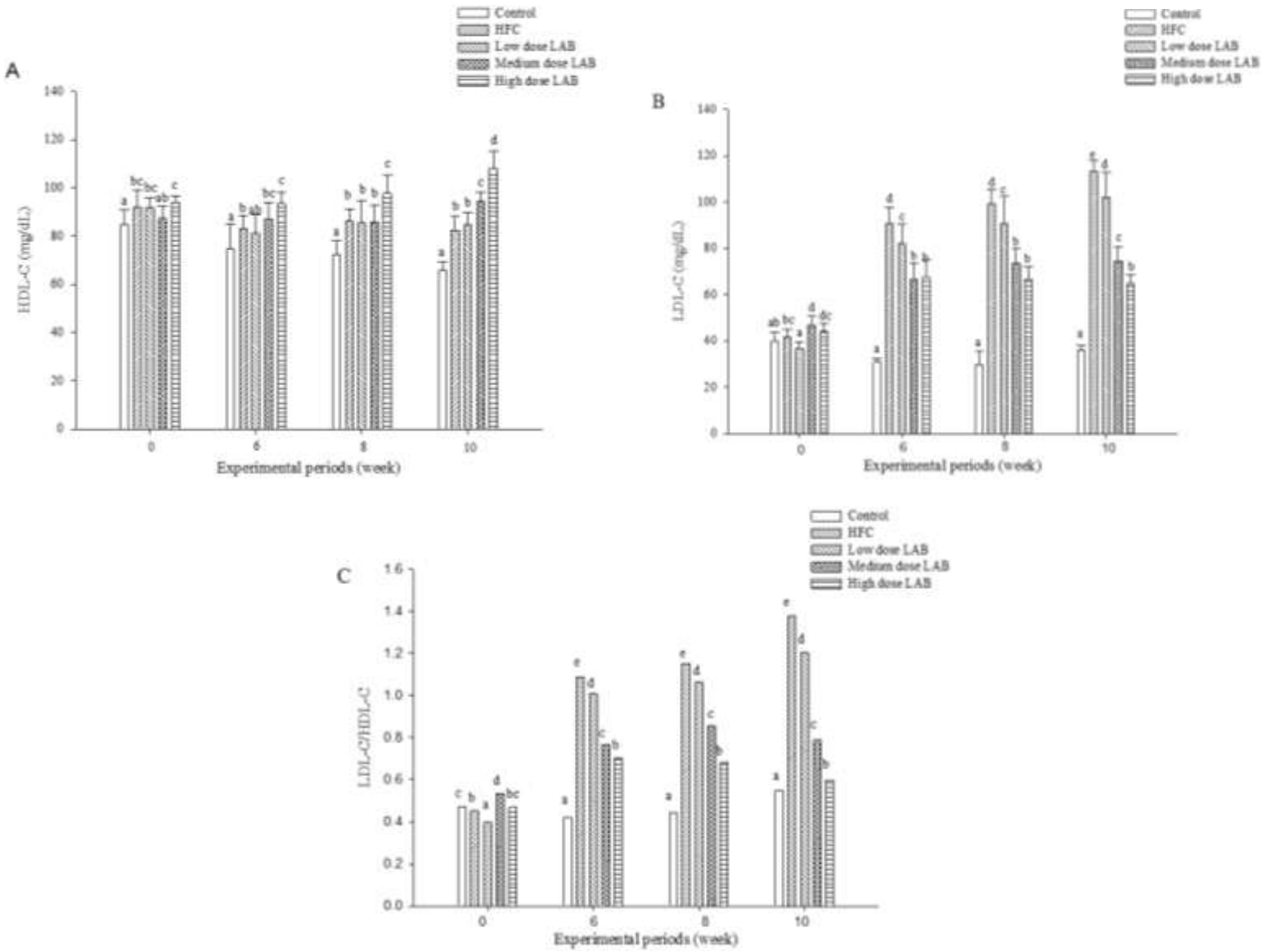

Figure 3. Serum (A) HDL-C, (B) LDL-C and (C) LDL-C/HDL-C ratio of hamsters fed a high-fat plus high-cholesterol diet and supplemented or not with different concentrations of probiotics complex product, PROBIO S-23, during 10 weeks of the study. Data are expressed as means ± SD (n=8). [a,b,c,d]Values in the same column with different superscripts mean significant difference ($P < 0.05$).

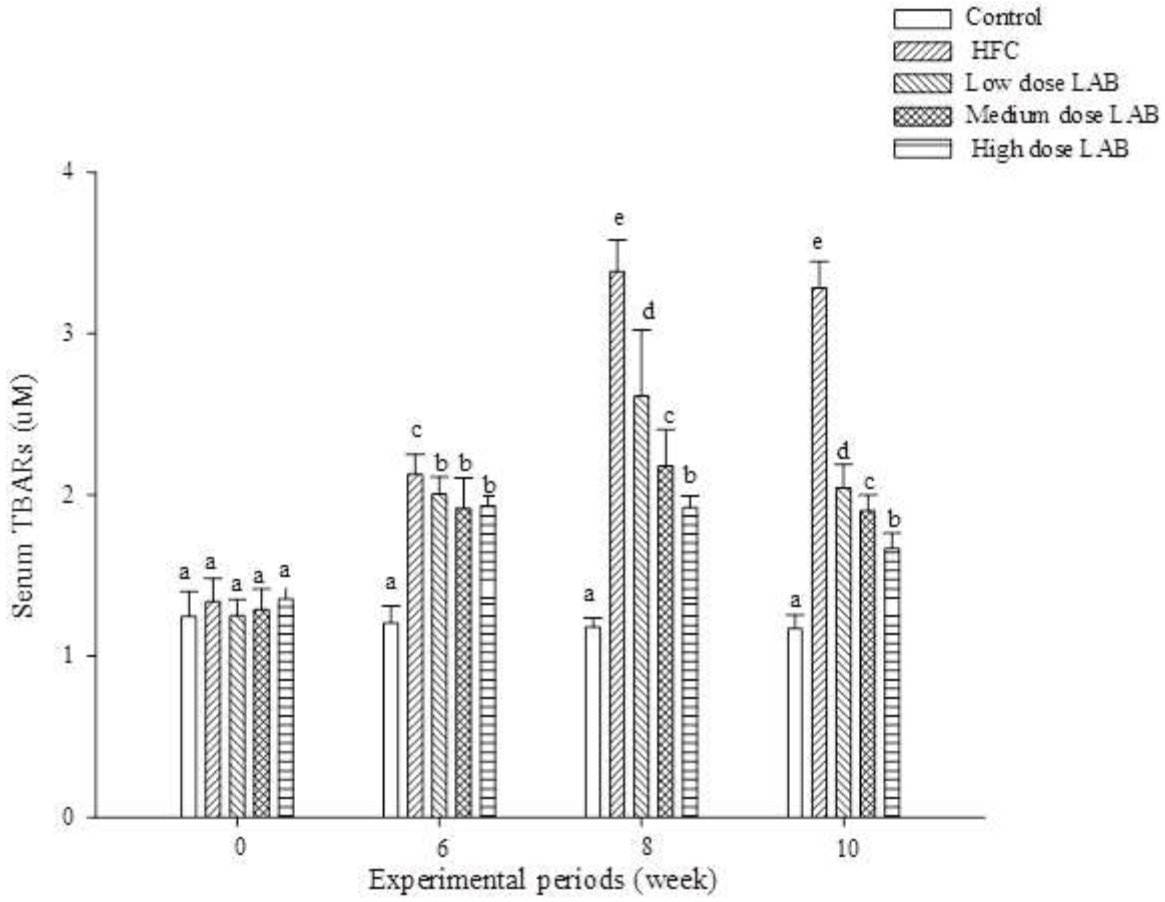

Figure 4. Serum lipid peroxides of hamsters fed a high-fat plus high-cholesterol diet and supplemented or not with different concentrations of probiotics complex product, PROBIO S-23, during 10 weeks of the study. Data are expressed as means ± SD (n=8). [a,b c,d,e]Values in the same column with different superscripts mean significant difference ($P < 0.05$).

(TBA) (Del Rio et al., 2005).

Yogurt was shown to have similar effects as we observed after administering PROBIOS-23. In the study conducted by Al-Sheraji et al. (2012), rats in the positive control group fed a cholesterol-enriched diet showed significant increase in MDA after 8 weeks. However, groups fed a cholesterol-enriched diet and supplemented with a yogurt containing *B. pseudocatenulatum* G4 or *B. longum* BB536 had significantly lower MDA levels than the positive control group after 8 weeks of treatment ($P < 0.05$).

Histopathological examination

After ten weeks of the experiment, the liver cells of hamsters in the HFC group, fed a high-cholesterol and high-fat diet, appeared empty, swollen, and even exhibited necrosis in some cases. In the high dose PROBIO S-23 group, although the tumescence of liver cells was not improved, the cavities and necroses observed were relatively few (Figure 5). Similar results were obtained by Hu et al. (2013), where histopathological examinations suggested severe injuries in liver tissues of rats fed high-cholesterol diet, while *L. plantarum* NS5 and NS12 strains partially ameliorated these injuries.

Conclusion

The results of our present and previous study (Tsai et al., 2014) indicate that PROBIO S-23 is a potential multiplex-strain probiotic product with the bile-salt hydrolase activity, and reduces the serum cholesterol, low-density lipoprotein cholesterol, malondialdehyde, and increases high-density lipoprotein cholesterol levels for the host.

Conflict of Interests

The authors have not declared any conflict of interests.

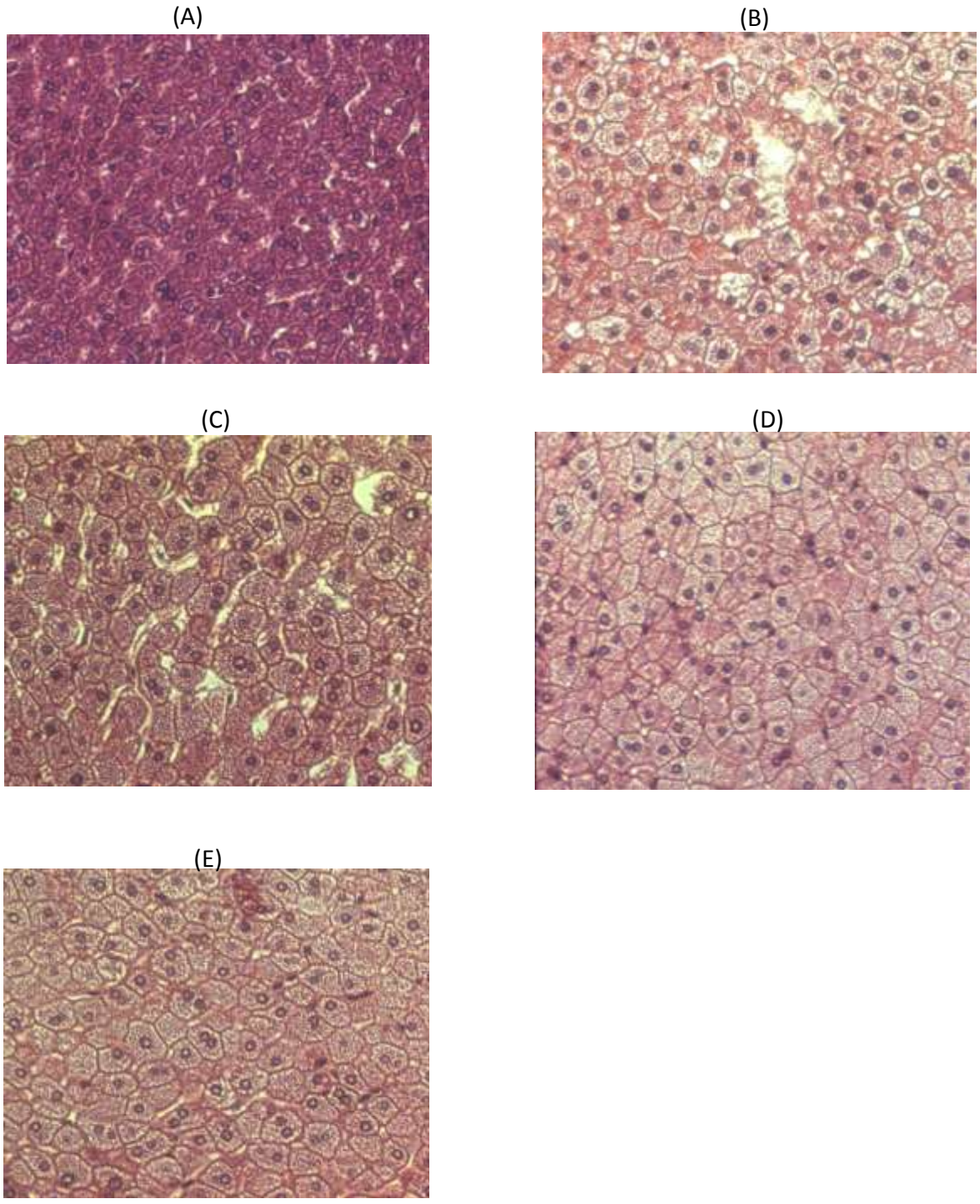

Figure 5. Histopathological changes in livers of hamsters fed a high-fat plus high-cholesterol diet and supplemented or not with different concentrations of probiotics complex product, PROBIO S-23, during 10 weeks of the study. (A) Control, (B) HFC, (C) Low dose LAB, (D) Medium dose LAB, and (E) High dose LAB (magnification 100×).

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Full Length Research Paper

Association study of single nucleotide polymorphism of human Toll like receptor 9 and susceptibility to pulmonary tuberculosis in Egyptian population

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Toll-like receptors (TLRs) are known to play important roles in human innate immune systems. Polymorphisms in and functions of TLRs have been investigated to identify associations with specific infectious diseases including tuberculosis (TB). This study was performed for 166 samples of unrelated individual's diagnosis of pulmonary tuberculosis and 98 household healthy samples. Genomic DNA was extracted from EDTA-anticoagulated peripheral blood. The alleles of (rs352140) TLR9 gene polymorphisms were detected using polymerase chain reaction restriction fragment length polymorphism. The resulting fragments were separated in 3% agarose gel electrophoresis. The sequence results generated by the forward and reverse sequencing primers were analyzed with the software program sequencing analysis. Sequence comparisons of three genotypes AG, AA, and GG were performed using the multiple-alignment algorithm in Megalign. The direct counting was used to determine the allele and genotype frequencies of each polymorphism. Hardy-Weinberg equilibrium (HWE) tests were performed in controls by Fisher exact test. Significant deviations from the Hardy-Weinberg equilibrium in the distribution of the TLRs SNP genotypes in TB patients and controls were not detected for the SNP TLR9rs352140 for both patients and controls. These results do not indicate a major influence of these putative functional TLR SNP on the susceptibility to (or protection from) tuberculosis in Egyptian population.

Key words: Association, TLR9, tuberculosis, Egyptian population.

INTRODUCTION

Tuberculosis (TB), an old and destructive disease, is a considerable public health problem. TB is an infectious disease caused by *Mycobacterium tuberculosis* (*Mtb*) (Jahantigh et al., 2013; WHO, 2010b). World Health

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Organization (WHO) evaluated one third of the population of the world is infected with *Mtb* (Dye et al., 1999). One billion people are infected in the 2000 to 2020, and about 200 million people will evolve active TB. Only 5 to 10% of the infected patients develop the active disease in their lifetime and 90% remain as latently *Mtb* infected individuals (Torres-Garcia et al., 2013). In spite the fact that Egypt is not found in the WHO list of 22 high TB infected countries, it is considered one of the high percentage in Eastern Mediterranean countries. In Egypt, TB is considered the third most important public health problem after schistosomiasis and hepatitis C (National Tuberculosis Control Program, 2006; WHO, 2010a). The National Tuberculosis Control Program of the Ministry of Health and Population (MOHP) in Egypt registers over 12,000 new TB patients every year. More than 50% of the cases are sputum smear-positive pulmonary TB (Helal et al., 2009). A previous study reported a co-infection with *Schistosoma mansoni* in every third hospitalized patient with tuberculosis in Tanzania. They suggested that schistosomiasis may reduce the host's immune response to the bacille calmette–guerin vaccine, which is widely used in endemic areas for protection against *Mtb*, and hence may lower the protective efficacy of the vaccine (Knopp et al., 2013). Furthermore, the joint effects of smoking, TB and human immunodeficiency virus (HIV) greatly increase the risk of chronic obstructive pulmonary disease in the long term (Ajagbe et al., 2014). The progression to active TB is the result of the environmental, host genetic factors and pathogenic characteristics of the *Mtb* strain (Torres-Garcia et al., 2013). Multiple genes have been involved in the control of *Mtb* and progression to TB (Pan et al., 2005; Yim and Selvaraj, 2010). Since a twin study established the importance of a genetic component as a factor in TB susceptibility (Comstock, 1978), linkage studies (Mahasirimongkol et al., 2009), genome wide association studies (Thye et al., 2010), and many candidate gene studies have been performed, and the candidate gene studies often focused on genes involved in immune function, including human leukocyte antigen (HLA) (Lombard et al., 2006), IL12B1 (Remus et al., 2004), Toll-like receptors (TLRs) (Velez et al., 2010), SLC11A1 (Van Crevel et al., 2009), IFNG (Pacheco et al., 2008), and CD209 (Vannberg et al., 2008).

TLRs are a family of phylogenetically conserved genes, that are essential for recognition of pathogen associated molecular patterns (PAMPs) on dendritic cells and macrophages (Azad et al., 2012; Bafica et al., 2005; Carvalho et al., 2011; Chen et al., 2010; Chow et al., 1999). TLR9, an endosomal localized receptor on B cells, plasmacytoid dendritic cells (pDCs), and monocytes/ macrophages, recognizes unmethylated nucleic acid motifs in bacterial and viral DNA (Hemmi et al., 2000). TLR9 is one of the most important receptors in the initiation of protective immunity against intracellular pathogens by activation signaling cascade of intracellular receptor signaling (Akira, 2006). TLR9 encoding gene is located on chromosome 3p21.3. It spans about 5 kb and contains two exons (Akira, 2006; Jahantigh et al., 2013). TLR-knockout mouse studies indicate that TLR2, TLR4, and TLR9 participate to host resistance to *Mtb* infection (Bafica et al., 2005). Genetic variations of TLR1, TLR2, TLR4, TLR6 and TLR9 have been associated with the susceptibility to TB in different ethnic groups (Ocejo-Vinyals et al., 2013; Thada et al., 2013; Zaki et al., 2012), but other studies have failed to demonstrate significant associations of TLRs polymorphisms with TB (Sanchez et al., 2012; Tian et al., 2013). Up to now, no previous studies have addressed the prevalence of TLRs polymorphisms in Egyptian patients with TB. Therefore, this study examined whether polymorphisms in TLR9 is associated with the susceptibility to pulmonary TB Egyptian individuals or not.

MATERIALS AND METHODS

Subjects

In this study, samples from 264 unrelated individuals were obtained. They were divided into 166 patient subjects with diagnosis of pulmonary TB collected from Abbassia Chest Hospital according to approval from health ministry in Egypt (Serial No. 09/2014) and 98 household healthy subjects as controls. This study included only subjects of 18 to 65 years old according to bioethics principles. The diagnosis of pulmonary TB was based on the WHO criteria with the presence of clinical symptoms, detection of acid-fast bacilli in sputum smear samples, *Mtb* positive cultures in Lwenstein-Jensen medium, X-ray evidence of cavitary lesions in lung. In the absence of clinical symptoms of active pulmonary tuberculosis, no medical history of TB, other infectious or autoimmune diseases, cancer and other diseases affecting host immunity were found in the control subjects. Informed consent was obtained from all subjects or their legal representatives before participation in the study. The protocol was IRB-approved at VACSERA-EGYPT Institutional Bioethics Review Board (BERD- VACSERA, EGYPT). A questionnaire including a full medical history regarding different variables as age, sex, residence, and medical history of TB was recorded. The study was explained to all participants using the consent form. TB outcome of adult subjects are to be tested by the current study, so subjects under 18 years old are not included. There is need to evaluate TB outcome in normal adult subjects whereas immunity of children, pregnant and immuno-compromised are out of our scope. About 3 to 5 ml EDTA-anticoagulated peripheral blood was drown from each subject and stored at -20°C.

DNA isolation and TLR9 genotyping

Genomic DNA was extracted from EDTA-anticoagulated peripheral blood using TIAN amp genomic DNA kit (Tiagen, Korea, Cat #DP304-02) according to its manufacturer instructions. The extracted DNA was stored at -20°C until further analysis. The alleles of (rs352140) TLR9 gene polymorphisms were detected using polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP). The amplification of TLR 9 (rs352140) fragments using PCR was performed according to Sambrook and Russel (2001) with some modifications using forward and reverse primers 5'-AAGCTGGACCTCTACCACGA-3' and 5'

Table 1. Demographic and clinical characteristics of the recruited subjects (patients and controls).

Comparison		Patient		Controls		*P* value
		No.	%	No.	%	
Gender	Female	46	27.8	46	46.9	0.0015**
	Male	120	72.2	52	51.1	
Age		38.2 ± 13.44		35.36 ± 7.86		0.057
Sputum analysis		143	86.1	0	0	
Sputum plantation		2	1.25	0	0	-
X-ray examination		166	100	0	0	
Diabetes		6	3.6	1	1.02	0.206
Hypertension		0	0	0	0	-
Autoimmune disease		0	0	1	1.02	0.193

TTGGCTGTGGATGTTGTT -3' (Pandey et al., 2011). PCR reaction was done using 200 ng of the template DNA molecule, 20 pmol of each primer and 2X PCR Master Mix (Tiagen, Korea) containing 10 mM dNTPs, reaction buffer [100 mM Tris - HCl pH 9.0, 500 mM potassium chloride (KCl) and 1% Triton X-100], 25 mM $MgCl_2$ and 0.25 µl Taq DNA polymerase [5 U/µl]. The cycling protocol using Thermal cycler PCR (Eppendorf, Germany) consisted of 35 cycles with strand denaturation at 95°C for 45 s, primer annealing at 56°C for 1 min, and primer extension at 72°C; 30 s for each stage. The PCR products were digested by restriction enzyme BstUI according to the manufacturer's instructions (New England Bio labs, UK). The resulting fragments were separated in 3% agarose gel containing 0.5 mg/ml ethidium bromide (Sigma, USA) by electrophoresis at 100 V for 30 min and visualized under UV trans illuminator.

Preparing sequencing cycle reaction

The deoxy ribonucleoside chain termination was employed for sequencing the double-stranded recombinant DNA obtained during the PCR procedure. Amplified DNA fragments were sequenced directly using the ABI Prism Big Dye Terminator V.3.1 Cycle sequencing Kit on an ABI 310 DNA automated sequencer (Applied Biosystems) .The reactions were done in 20 µl mixture reaction, according to the manufacturer's instructions. Sequencing cycle for SNP at TLR9 rs352140 was performed with the following parameters: initial denaturation of 95°C for 5 min, 35 cycles of 95°C for 45 sec, 56°C for 1 min, and 72°C for 30 sec, followed by a final extension of 72°C for 7 min. But sequencing cycle was performed with the following parameters: preheating at 95°C for 1 min, 35 cycles of 95°C for 1 min, 47°C for 1 min, and 72°C for 1 min; a final 72°C for 10 min as a final extension step (Nguyen et al., 2009).

Removal of unincorporated dye terminator

DyeEx Kits for purification was used to remove all unincorporated dye terminator directly from sequencing reaction. Spin columns were gently vortexed to re-suspend the resin using centrifugation for 3 min at 750 ×g. The sequencing reaction 20 µl was slowly applied to the gel bed and centrifuged for 3 min at 750 ×g. The samples were dried in a vacuum centrifuge (Van Houdt et al., 2010).

Sequencing

Sequencing was carried out at Genetic Engineering Research Department (Vacsera, Egypt). After removing all the unincorporated dye terminator as mentioned earler, all samples have been re-suspended in Hi Di-ionized formamide, denatured at 95°C for 3 min and 30 s, then applied on chilled ice for 5 min. Electrophoresis process was performed on ABI Prism 310 Genetic Analyzer, by using ABI Prism 310 collection data base. All samples were analyzed using sequencing analysis software. The sequence results generated by the same forward and reverse sequencing primers of PCR were analyzed with the software program sequencing analysis v5.3.1. For sequence comparisons of three genotypes AG, AA, and GG, sequence alignment was performed using the multiple-alignment algorithm in Megalign (DNASTAR, Window version 3.12e). Also, the resulted sequence was aligned with National Center for Biotechnology Information (NCBI).

Statistical analysis

The direct counting was used to determine the allele and genotype frequencies of each polymorphism. Hardy–Weinberg equilibrium (HWE) tests were performed in controls by Fisher exact test. A P-value of less than 0.01 was considered to indicate deviation from HWE. Association analysis between SNPs and TB was also performed by Fisher exact test. A P-value of less than 0.05 was considered to be statistically significant. Odds ratio (OR) with 95% confidence interval (CI) were calculated for the SNP for evaluating the relative risk using Statcalc program (Graphpad prizm version 5.0).

RESULTS

Demographic and clinical characteristics of TB patients and controls are summarized in Table 1. One hundred and sixty six patients with TB and 98 controls were investigated for the presence of the TLR9 polymorphisms. The demographic characteristics of the 166 patients with TB participating in this study show that they had a mean age (± standard deviation) of 38.2 ± 13.44. The result shows 86.1% of the TB patients were sputum positive and 6.3% had diabetes mellitus. TB infection was confirmed using X-ray examination for both patients and controls as shown in Table 1. Notably, none of the subjects reported any history of other clinical symptoms

Table 2. Contribution of the TLR9 C2848T (rs352140) polymorphisms in Egyptian patients with pulmonary tuberculosis.

Genotype	Patients (frequency)	Control (frequency)	χ^2	*P* value	Odds ratio (95% CI)
GG	43 (25.9)	19 (19.4)	1.46	0.23	1.45
GA	81 (48.8)	53 (54.1)	0.74	0.39	1.17
AA	42 (25.3)	26 (26.5)	0.05	0.83	0.94

Table 3. Assessment of gender in relation to TLR9 C2848T (rs352140) polymorphisms in study group.

Parameter	Genotype	Male (Frequency, %)	Female (Frequency, %)	χ^2	*P* value	Odds ratio (95% CI)
Patient	GG	34 (28.3)	9 (19.6)	1.33	0.25	0.62
	GA	56 (46.7)	25 (54.3)	0.64	0.42	0.82
	AA	30 (25.0)	12 (26.2)	0.02	0.89	1.06
Controls	GG	8 (15.4)	11 (23.9)	1.14	0.29	1.73
	GA	27 (51.9)	26 (56.5)	2.16	0.14	0.50
	AA	17 (32.7)	9 (19.6)	2.31	0.12	1.55

at the time of sampling. Mean age, gender and the prevalence of other comorbidities such as systemic hypertension, immunodeficiency and autoimmunity were similar among groups.

Using PCR-RFLP, the observed genotype and allele frequency distribution of TLR9 C2848T (rs352140) gene polymorphisms between Egyptian patients with pulmonary tuberculosis and controls are illustrated in Table 2. Significant deviations from the Hardy-Weinberg equilibrium in the distribution of the TLRs SNP genotypes in TB patients as well as controls were not detected for the SNP TLR9rs352140 in both TB patients and controls. The TLR9 C2848T (rs352140) genotypic and allelic frequency distributions between Egyptian patients with pulmonary tuberculosis and healthy controls were similar and no significant association was observed for pulmonary tuberculosis development ($p > 0.05$). When univariate analysis was performed by age, gender and clinical data, no statistical difference was observed ($p > 0.05$) between healthy and patient subjects in all criteria except for gender which is in accordance with published data indicating that in most low-income countries, the ratio of male to female cases of tuberculosis is approximately 2:1 (WHO, 2004) attributable to biological characteristics and socioeconomic and cultural barriers to access healthcare (Borgdorff et al., 2000). Table 3 illustrates genotype and allele frequency distribution of TLR9 C2848T (rs352140) gene polymorphisms between male to female in the study group and healthy control subjects indicating no significant differences based on gender difference.

Figure 1 shows the digested PCR products with BstUI digestion for TLR9 rs-352140. DNA bands at 135 and 42 bp corresponded to the homozygousTLR9 GG genotype, while bands of 177, 135 and 42 bp were designated as heterozygous GA; a band of 177 bp corresponded to the homozygous AA genotype. Digested samples were separated by electrophoresis on 3% agarose gel and visualized by ethidium bromide staining.

Three random samples from each genotype were chosen for sequencing to confirm TLR9 rs352140 SNP detection using RFLP-PCR assay. Figure 2 shows the sequencing results within the TLR9-TIR. All the sequencing results matched with the RFLP-PCR assay results. Direct sequencing of the 177-bp region containing the single nucleotide polymorphism site of interest confirmed the genotyping results at nucleotide 135. The resulting sequence was: AGCTGAGGTCCAGGGCC TCCAGTCG[C/T]GGTAGCTCCGTGAATGAGTGCTCG.

To confirm these results, the sequence alignment was done in NCBI blast. The Identities was 50/50 (100%) (Figure, 3).

DISCUSSION

TB caused by *Mtb*, is a main health problem worldwide, with about 10 million new patients diagnosed every year. Innate immunity plays an important role in the host defense against *Mtb*. The recognition of *Mtb* by cells of the innate immune system is the first step in this process. Some human epidemiological studies detected that genetic variation in genes encoding for recognition receptors (PPRs) and downstream signaling products influence disease susceptibility, severity, and outcome. TLRs are a family of PRRs consisting of 12 members in

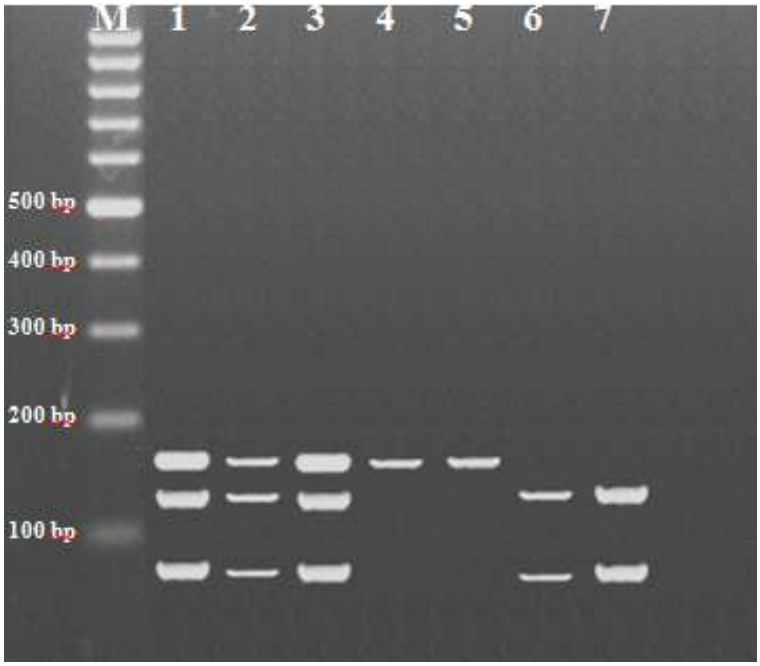

Figure 1. Representative agarose gel image of digested PCR products with restriction enzyme BstU1 for TLR9 rs-352140 (M:100-bp ladder). (a) Genotype GA (heterozygous carrier of polymorphism) showing bands of 177, 135 and 42 bp; represented in lanes 1,2 and 3. (b) Genotype AA (homozygous carrier of polymorphism, undigested PCR product of 177bp; represented in lanes 4 and 5. (c) Genotype GG (normal) showing bands of 135 and 42 bp; represented in lane 6 and 7.

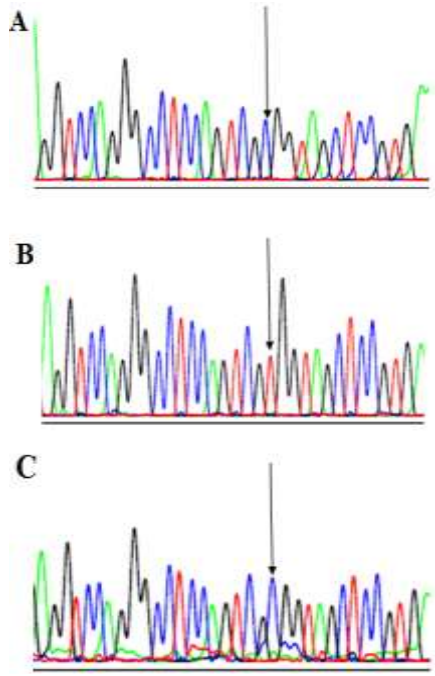

Figure 2. Sequencing results of TLR-9 rs352140 genomic DNA. (A) Homozygous (GG) genotype; (B) Homozygous (AA) genotype (C) Heterozygous (GA) genotype.

human and other mammals. TLRs expression is carried out on the surface of the cell membrane or on the membrane macrophages and dendritic cells. In spite of the interaction of *Mtb* with TLRs leads to phagocyte activation, the interaction itself does not lead to immediate ingestion of the mycobacteria (Kleinnijenhuis et al., 2011). Host genetic may involve multiple genes and their polymorphisms to develop TB (Leandro et al., 2013). TLR association may differ from population to another. For example, TLR2 polymorphisms are not responsible for the increased prevalence of TB in the Indian population. While significant TLR2 gene has been reported exclusively in the Caucasian population, a Korean population and in a Tunisian population. Other authors have failed to detect these polymorphisms in the Korean population (Biswas et al., 2009).

Previous studies revealed that TLR9 has critical role in the incidence of TB. The gene of TLR9 is located on chromosome 3p21.3. The total length of TLR9 gene is approximate 5 kb. Its coding gene has two exons, and the main coding region is in the second exon (Chen et al., 2015). So, the objective of the current study was to detect the presence of association between TLR9 C2848T (rs352140) polymorphism and tuberculosis infection among patients with tuberculosis in Egyptian population. This study found that there were no significant differences between neither male and female pulmonary

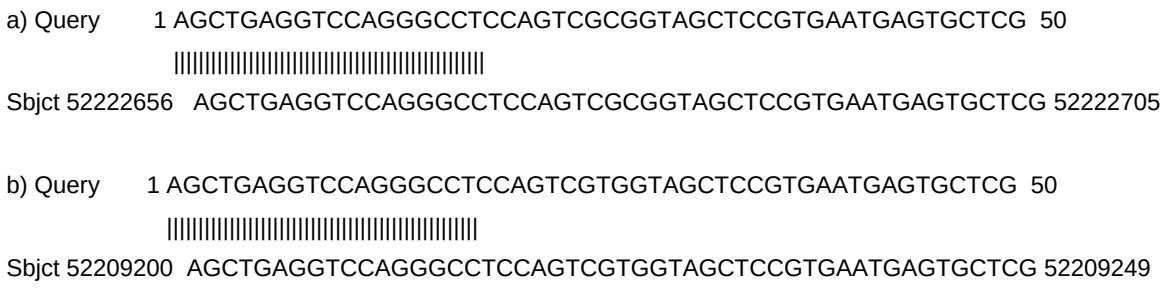

Figure 3. Represents the identities of the resulted TLR9 sequencing corresponding genes of NCBI results. Where (a) Sequence ID: ref|NC_000003.12|Length: 198295559. (b) Sequence ID: ref|NC_018914.2|Length: 197992941.

TB nor control groups for genotype frequencies regarding TLR9 C2848T (rs352140) polymorphism. In agreement with these findings, Salimi (2015) revealed that TLR8 rs3764880 and TLR9 rs148805533 polymorphisms may not be risk factors for susceptibility to pulmonary tuberculosis in a sample of Iranian population. In their study, they found no association between polymorphism and pulmonary tuberculosis neither in their female nor in their male patients. In addition, they did not observe the Del allele of 14 bp Ins/Del polymorphism of TLR9 gene in the studied population and reported no association between this polymorphism and pulmonary tuberculosis. In another study, Iranian population was performed in 124 newly diagnosed TB cases and 149 healthy controls in a TB-endemic region of Iran. They found that no significant relation between TLR4 and TLR9 polymorphisms alone and TB (Jahantigh et al., 2013). Also, similar to the current results, there was no association between TLR9 rs148805533 (14 bp Ins/Del) polymorphism and TB in South India (Selvaraj et al., 2010). Other candidate gene studies have examined the relationship between TLR9 SNPs and pulmonary TB. In previous study, a meta-analysis was performed to assess the association between seven extensively studied TLR9 polymorphisms (rs187084, rs352165, rs5743836, rs5743842, rs352139, rs352140 and rs352167) and TB risk. The analysis revealed an association between certain TLR9 polymorphism and TB risk. In addition, 5 different genetic models (Allele, Heterozygote, Homozygote, Dominant and Recessive model) were analyzed in all polymorphisms. A subgroup analysis by race was also performed for rs187084, rs352139 and rs5743836 polymorphisms, the studies included Indians, Iranian and West African, Indonesians, Vietnamese, Chinese and Mexicans. The results showed that rs187084 and rs5743836 polymorphisms were not associated with TB risk, while the association between rs352139 polymorphism and TB risk may vary by race (Chen et al., 2015). Also, expression of TLR7, TLR8 and TLR9 was determined in monocytes from HIV-infected patients and control subjects, which were activated with specific ligands. The expression of MyD88 and NF-kBp65 were determined by flow cytometry. No statistical difference was found in the expression of TLR7, 8 and 9 in monocytes from patients compared to controls, but they observed the non-significant increased expression of TLR9 in patients (Valencia et al., 2013). In an animal trial, the relation between TLR1 and TLR9 with TB susceptibility in Chinese Holstein cattle was examined. They suggested that variants in the TLR1 gene are associated with susceptibility to TB, whereas no significant association can be inferred from the polymorphisms in the TLR9 gene (Sun et al., 2012). In another study to investigate the role of TLR9 in innate immunity to *Mycobacterium avium*, TLR9, TLR2, and MyD88 knockout (KO) mice were infected with this bacterium. They proved that TLR2 and MyD88, but not TLR9, played a major role in interleukin-12 and TNF-α production by *M. avium*-infected macrophages and dendritic cells (DCs). They also found that major histocompatibility complex class II molecule expression on DCs is regulated by TLR2 and MyD88 signaling, and not by TLR9 (Carvalho et al., 2011).

On the contrary, many researchers proved that TLR genes have been variably associated with tuberculosis infection and there is strong evidence indicating that host genetic factors play critical roles in tuberculosis susceptibility, severity and development (Azad et al., 2012; Davila et al., 2008; Khalilullah et al., 2014). It was suggested that the allele A of the intronic polymorphism rs352139 on TLR9 gene might contribute to the risk of developing TB in Mexican Amerindians. Polymorphisms and functions of TLRs have been investigated to identify associations with specific infectious diseases, including TB. A statistically significant association was observed between TB susceptibility in a classified Indonesian female group and rs352139, an SNP located in the intron of TLR9 (Torres-Garcia et al., 2013). Meta-analysis of the Indonesian and Vietnamese populations showed that rs352139 was significantly associated with TB in the recessive model. This finding indicated that a TLR9 polymorphism might have an important role in the susceptibility to *Mtb* in Asian populations (Kobayashi et al., 2012). In Vietnam, using a case population design,

they evaluated whether SNPs in the TLR9 gene region were associated with susceptibility to pulmonary or meningeal TB as well as neurologic presentation and mortality in the meningeal TB group (Graustein et al., 2015). It was found to be essential for cellular responses to mycobacterial CpG DNA. *In vitro* studies showed that DCs release IL-12 in response to *Mtb* through TLR9 (Thada et al., 2013). A report demonstrates that TLR9-deficient mice are susceptible to *Mtb* infection, and mice lacking both TLR2 and TLR9 are more susceptible to TB (Bafica et al., 2005). In a study in three independent population samples indicate that variations in TLR2 and TLR9 might play important roles in determining susceptibility to TB in African-Americans population when TLR1, TLR2, TLR4, TLR6, and TLR9 were examined (Velez et al., 2010). In another study in India, it is found, a significantly lower minor allele frequency (MAF) of T-1486C in the Baiga tribe, wherein fewer PTB cases were reported, than that in the Gond and Korku tribes. These data suggest that the minor "C" allele at rs187084 locus may be associated with susceptibility to PTB (Bharti et al., 2014).

Conclusions

The results of this study indicate that the single nucleotide polymorphisms in TLR9 (SNP TLR9 rs352140) gene might not be associated with TB risk in Egyptian population.

Conflict of Interests

The authors have not declared any conflict of interest.

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Full Length Research Paper

Bacteriological and molecular studies on *Clostridium perfringens* isolated from sheep in three Egyptian provinces

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The study was intended to determine types of *Clostridium perfringens* and their toxins in diseased sheep with suspected enterotoxaemia, apparently healthy in contact sheep and soil in three Egyptian provinces (one year study). A total of 800 sheep were visited regularly over a period of one year to record cases of enterotoxaemia and collection of samples for bacteriological examination and toxin genotyping using multiplex polymerase chain reaction (PCR) by using four primers set specific for genes encoding toxin production (alpha, beta, epsilon and iota). Based on bacteriological examination, the percentage of *C. perfringens* isolated from soil, apparently healthy, and diseases sheep were 41, 12 and 59%, respectively. The results of multiplex PCR indicated that *C. perfringens* type A was the predominant followed by *C. perfringens* type D with an incidence rate of 43 and 42.7% from positive samples, respectively. While, *C. perfringens* type B was successfully recovered only from 14.61% of positive samples. Worth mentioning, the data presented collectively highlighted the role of soil and apparently healthy as a potential source of re-infection. Moreover, it is recommended that *C. perfringens* type A should be included in vaccine schedule in order to afford adequate protection and lessen the adverse economic loses of sheep clostridial diseases.

Key words: Clostridia, sheep enterotoxaemia, multiplex polymerase chain reaction (PCR), Egypt.

INTRODUCTION

Sheep and goat are considered as one of the important pillars of income in many developing countries including Egypt. Thus, sheep diseases especially that caused by *Clostridium perfringens* most notably sheep enterotoxemia

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are considered the most risk factor opposite this industry (Ahmed, 2004).

C. perfringens is a normal inhabitant of the intestine of most animal species, present in a small number and produce a little amount of toxin easily removed by the normal gut movement, but when the intestinal environment is altered by sudden changes in diet or other factors, *C. perfringens* proliferates and produces potent toxins that act locally or are absorbed into the general circulation with usually devastating effects on the host (Uzal and Songer, 2008). The diseases are generally called enterotoxemia in sheep, goats and other animals (Uzal and Songer, 2008).

C. perfringens is classified into 5 major toxin types (A, B, C, D, and E) on the basis of expression of 4 major toxins: alpha, beta, epsilon, and iota (Gokce et al., 2007). The clinical manifestation and severity of the infection in correspondence varies markedly according to toxigenic type involved and the specific toxin produced (Greco et al., 2005).

Enterotoxemia is one of the most frequently occurring diseases of sheep and goats worldwide. Reports from countries around the world have reported prevalence rates of enterotoxaemia ranging between 24.13 and 100% (el Idrissi and Ward, 1992; Fayez et al., 2013).

Presumptive diagnosis mainly depends on clinical signs and gross post-mortem findings. In addition, identification of the toxins in intestinal content is crucial for accurate diagnosis. Traditional methods of toxins identification are mainly based on neutralization test in mice or skin of guinea pigs. In the last decades, Enzyme-linked immunosorbent assay (ELISA) kits have been used for the detection of Clostridial toxins (Uzal et al., 2003). DNA-based techniques, such as polymerase chain reaction (PCR), have been developed for *C. perfringens* genotyping and are a reliable alternative method to testing in laboratory animals (Baums et al., 2004).

To the author knowledgement, only a few studies were conducted to estimate the incidence of *C. perfringens* toxin in camels and humans (Habashy et al., 2009; Mohamed et al., 2010). Moreover, there is a scarcity of data regarding the toxigenic type of *C. perfringens* predominantly implicated in sheep enterotoxaemia in Egypt. In addition, the role of soil and apparently healthy sheep is still unknown.

Thus, this study was undertaken to estimate the incidence of *C. perfringens* induced sheep-enterotoxaemia in three Egyptian provinces using conventional bacteriological methods and multiplex PCR for elucidating the correct toxigenic type.

MATERIALS AND METHODS

Clinical and necropsy finding

A total of 20 sheep flocks located at Desert Road, Waedielnatron, Merkz bader and Sadat city were regularly visited for one year to inspect the animals for clinical signs of enterotoxaemia. Each flock was examined clinically according to Radostitis et al. (2007). Clinical symptoms of clinically diseased sheep were recorded. Necropsy findings of the dead animals were recorded according to Griffiths (2005) within 6 h of death. Samples for bacteriological examination were taken from rectum and intestinal contents of sheep which displayed a variety of clinical signs such as abdominal pain, bloat, mild to severe (blood-tinged to bloody) diarrhea and recumbence. Small intestine specimens (duodenum) were rapidly taken from recently dead or sacrificed animals with clinical symptoms of dysentery, in order to avoid non-specific *C. perfringens* (Osman, 1993; Ahmed, 2004). In some animals, nervous symptoms (dullness, muscular tremors, opisthotonos and convulsions) were observed, while in other cases death was the only clinical sign observed.

Histopathological examination

Specimens from brains, kidneys, livers and small intestines were collected after necropsy and fixed in 10% buffered neutral formalin solution. Five-micron thick paraffin sections were prepared, stained with Hemotoxylin & Eosin stain (H&E), and then examined microscopically for histopathology (Bancroft and Gamble, 2002).

Samples for bacteriological examination

Four hundred (400) samples were collected from the soil of sheep farms, clinically healthy and sheep with enterotoxemia for isolation of *C. perfringens* (Table 1). Regarding soil samples, about 10 g of soil were collected in sterile plastic bags after removal of the superficial layer of the soil at a depth of 20 cm and sent directly to a laboratory for bacteriological isolation followed by toxin identification using multiplex PCR. Briefly, the fecal samples were collected from the rectum of diseased sheep and healthy in contact sheep and directly sent to laboratory for bacteriological examination. While, in case of intestinal samples, portions of small intestine (congested and/or ulcerated) from diseased and healthy in contact sheep were ligated from both ends, cut and put in sterile plastic bags and directly sent to laboratory in cold ice (Osman, 1993; Ahmed, 2004).

Isolation and identification of *C. perfringens* (Willis, 1977)

C. perfringens was isolated on cooked meat medium (Oxoid), 10% sheep blood agar (Oxoid), Perfringens agar medium LAB 194 (Lab M): Typical colonies were identified as described by Murray et al. (2003), and full identification of the isolates mainly depending on their characteristic colonial morphology, hemolysis activity, Gram staining and biochemical tests according to Cruickshank et al. (1975), Koneman et al. (1988) and Quinn et al. (2002).

Multiplex PCR

The whole genomic DNA was extracted according to Sambrook and Russell (2001). Briefly, 1 ml of overnight culture in fluid thioglycollate broth (FTB) supplemented with D–cycloserine was centrifuged for 20 min at 13,000 rpm. The pellets were then re-suspended in 600 µl of ice cold cell lysis buffer. Then 3 µl of proteinase K solution was added and incubated at 55°C for 3 to 16 h to increase the yield of genomic DNA. Finally, the extracted DNA pellet was stored in 100 µl of tris-EDTA buffer at -20°C for further analysis. The PCR reactions conditions were carried out using four

Table 1. Types and numbers of samples collected for bacteriological examination.

Types of samples collected		Number
Soil	Villages area	50
	Newly reclaimed area	50
Examined suspected sheep	Faecal samples	70
	Intestinal contents	130
Apparently healthy sheep	Faecal samples	50
	Intestinal contents	50
Total		400

Table 2. The nucleotide sequences of primers used for multiplex PCR amplification (Greco *et al*., 2005).

Toxin/Gene	Oligonucleotide sequences	Fragment length (bp)
α/cpa	5-TGC TAA TGTTAC TGC CGT TGA TAG-3 5-TGC TAA TGTTAC TGC CGT TGA TAG-3	247
β/cpb	5-AAC TTA ACT GGA TTT ATG TCT TCA-3 5-ATA GTA GAA AAA TCA GGT TGG ACA-3	317
ε/etx	5-ATT AAA ATC ACA ATC ATT CAC TTG-3 5-CTT GTG AAG GGA CAT TAT GAG TAA-3	206
í/iap	5-TTT TAA CTA GTT CAT TTC CTA GTT A-3 5-TTT TTG TAT TCT TTT TCT CTA GGA TT-3	298

primers sets as described previously (Greco et al., 2005) (Table 2). The reactions were performed in 50 µl volumes in PCR tubes. The reaction mixture consists of 10 µl DNA template, 5 µl of 10 × Dream Taq green buffer, 3 µl of dNTPS (10 mM of each), 0.5 µl of Dream Taq polymerase (2.5 u), 1 µl of each primer (50 pmoles/µl) and 23.5 µl of DNA-free water. The thermal conditions were 95°C for 5 min followed by 35 cycles consisting of 1 min of denaturation at 94°C, 1 min of annealing at 50°C and 1 min of extension at 72°C. The final extension was performed at 72°C for 10 min. The PCR products were analyzed using 1.5% agarose gel and stained with ethidium bromide. Amplified bands were visualized and photographed under UV illumination.

RESULTS

Clinical symptoms

Two hundred sheep out of the 800 (25%) examined sheep showed signs of clostridial enterotoxemia, with a mortality rate of 16.25% (130/800). In addition, 80 diseased sheep out of 200 (40%) showed peracute and acute forms of illness (sudden death to death within 24 h), while 120 (60%) of the diseased sheep showed sub-acute illness (death occurs within 72 h from the beginning of illness). Three clinical forms had been identified; the first form is characterized by acute onset of depression, pale mucus membrane, collapse, dyspnea and severe abdominal pain. This form ended by the death of diseased animals within the same day of onset of illness while few animals may survive for several days. The second form is characterized by sudden death. In some cases, depression, with abdominal pain and severe bloody to greenish diarrhea were noticed. The last observed form is characterized by diarrhea, inappetence, depression ended by collapse and death. In some cases, progressive weakness with the development of nervous signs such as dullness, ataxia, incoordination and convulsive movement of the head with the neck rest laterally on the shoulder were observed.

Necropsy finding

The necropsy findings of the examined cases were summarized (Figure 1A, B, C and D). The post-mortem

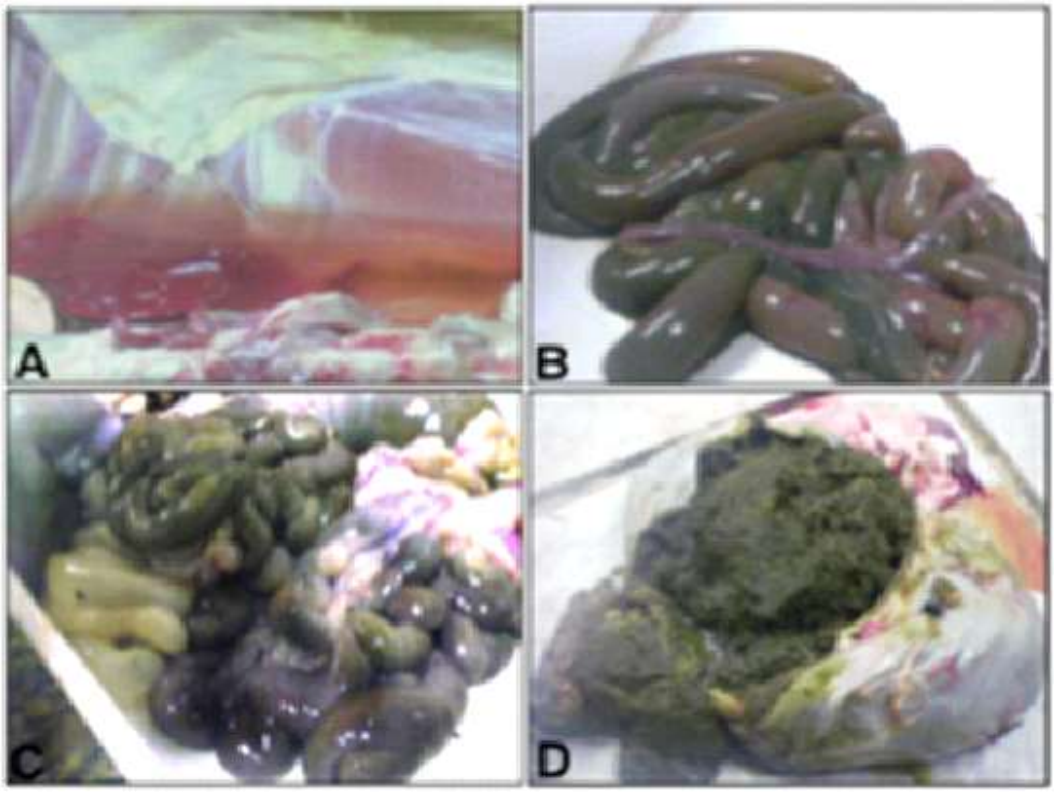

Figure 1. Necropsy findings of the diseased sheep. A) Bloody fluids in body cavities. B) Severely congested part of sheep small intestine. C) Severely congested sheep small intestine with gases. D) Rumen of sheep overloaded with ingesta.

examination of dead cases of the three forms showed bloody fluid in body cavities (Figure 1A) with flappy heart, severely congested intestine (Figure 1B), the presence of gasses in the small intestine with the overloaded rumen (Figure 1C to D), congestion of brain and meninges with friable congested liver and kidneys. Of note, there is no post-mortem changes that could be reported in some cases.

Histopathology

The histopathological changes showed severe necrosis and destruction of intestinal villi, hemorrhages, edema in the intestinal lamina propria with severely congested blood vessels (Figure 2A and B). The liver showed dissociation of hepatic cords, hemorrhages, congestion of central vein, edema under the endothelial lining of a hepatic sinusoid with portal edema and coagulative necrosis (Figure 2C and D). The kidneys showed hyper-cellularity with red blood cells infiltration in Bowman's space, medullary hemorrhages and nephrosis with necrosis of proximal and distal convoluted tubules (Figure 2E, F and G). Malacia, demyelination, perivascular hemorrhages, edema and congestion of both cerebrum and cerebellum were also observed in brain samples.

Isolation and identification of *C. perfringens*

C. perfringens was isolated and characterized based on typical colony morphology on sheep blood agar with a characteristic double zone of hemolysis, while on perfringens agar medium supplemented with D-cycloserine and egg yolk emulsion, the micro-organism appeared as small black colonies surrounded by halo area due to lecithinase activity. Gram-stained smear from the colonies revealed the typical appearance of Gram-positive straight sided rods arranged singly or in pairs. The percentage of *C. perfringens* isolation from the soil, apparently healthy and diseased sheep were 41% (41/100), 12% (12/100) and 59% (118/200), respectively.

Multiplex PCR for clostridia toxin type

Depending on the results of PCR and toxin genotyping, the data presented in Table 3 showed that the percentage

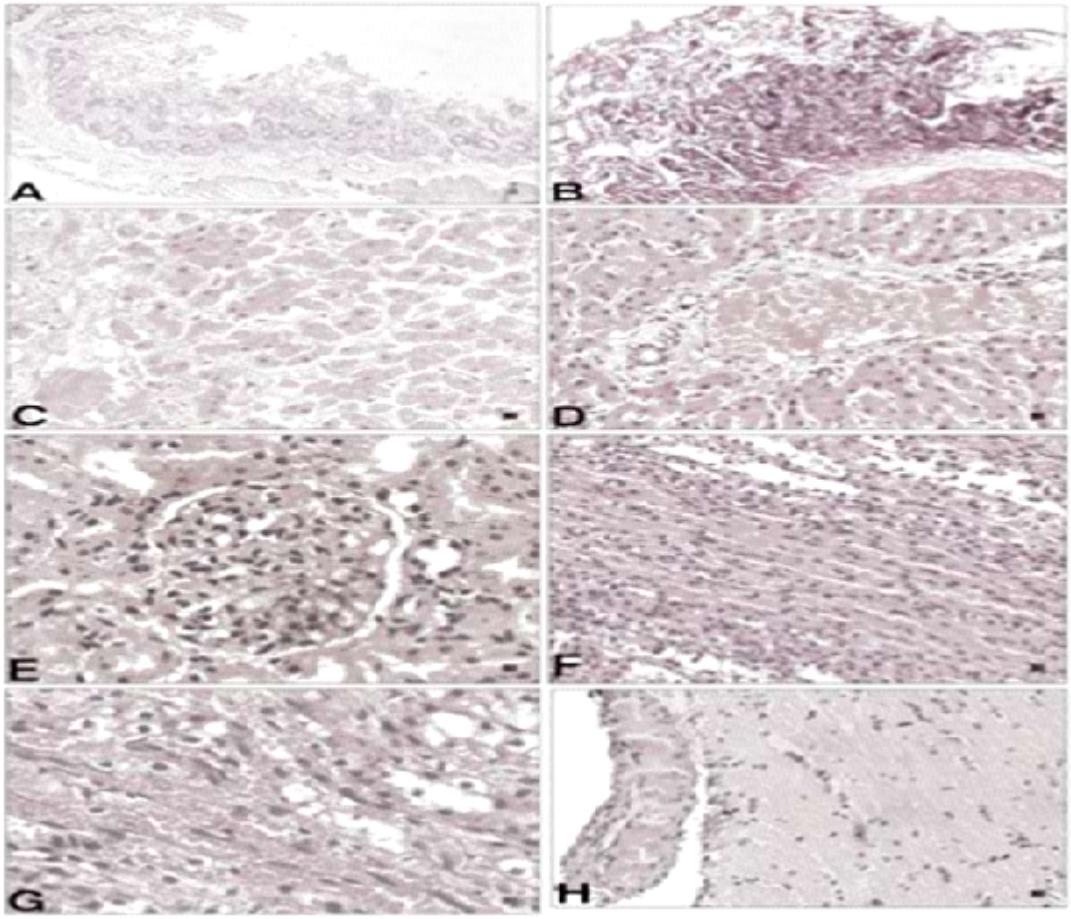

Figure 2. Histopathological examination of enterotoxemic sheep. (A) Sever necrosis and destruction of intestinal villi haemorrhages, oedema in lamina propria with severely congested blood vessels H&E (x4). (B) Sever necrosis and destruction of intestinal villi with haemorrhages H&E (x10). (C) Dissociation of hepatic cord with coagulative necrosis H&E (x20). (D) Congestion in central vein, hepatic hemorrhages, dilation in hepatic sinusoid with edema under the endothelial lining H&E (x20). (E) Dilatation of Bowman's space with red blood cells infiltration and necrosis in convoluted tubules H&E (x40). (F) Medullary haemorrhages with necronephrosis H&E (x20). (G) Haemorrhages, vacuolation and destruction in distal convoluted tubules H&E (x40). (H) Capillary congestion with edema in cerebellum H&E (x20).

of *C. perfringens* type A (cpa gene only), *C. perfringens* type B (cpa, cpb and etx genes) and *C. perfringens* type D (cpa and etx genes) in positive soil samples was 70.37% (29/41), 7.31% (3/41), and 21.95% (9/41), respectively. While in case of the apparently healthy sheep, the incidence rate of *C. perfringens* type B (cpa, cpb and etx genes) and *C. perfringens* type D (cpa and etx genes) in positive samples was 50% (6/12) and 50% (6/12) correspondingly, with no positive samples for *C. perfringens* type B. Finally, a higher incidence was observed in diseased sheep, *C. perfringens* type A (cpa gene only), *C. perfringens* type B (cpa, cpb and etx genes) and *C. perfringens* type D (cpa and etx genes) were recovered from 35.59% (42/118), 21.18% (25/118), and 45.76% (54/118) of positive samples in that order. The overall incidence showed that, *C. perfringens* type A (cpa gene only), *C. perfringens* type B (cpa, cpb and etx genes) and *C. perfringens* type D (cpa and etx genes) were recovered from 45.03% (77/171), 14.61% (25/171), and 40.35% (69/171) of the positive clostridial perfringens (Table 3).

DISCUSSION

C. perfringens is spore-forming Gram-positive cocci that produce more than 17 toxins (Li et al., 2013; Popoff and Bouvet, 2013). *C. perfringens* is classified into five main groups A to E in relation to the production of four lethal toxins alpha, beta, epsilon and iota toxins (McCourt et al.,

Table 3. Prevalence of *C. perfringens* recovered from diseased, apparently healthy sheep and soil and toxin genotyping using multiplex PCR.

Type of isolate	*C. perfringens* Type A		*C. perfringens* Type B		*C. perfringens* Type D		Total
Source of samples	No	%	No	%	No	%	
Village soil	11	84.62	0	0	2	15.38	13
Newly reclaimed soil	18	64.28	3	10.72	7	25	28
Clinically healthy sheep	6	50	0	0	6	50	12
Diseased sheep	42	35.59	25	21.18	54	45.76	118
Total	77	45.03	25	14.61	69	40.35	171

2005; Gurjar et al., 2008). *C. perfringens* induced enterotoxemia in sheep and goat represents a major economic obstacle facing developing countries attributable to the high fatality rate, decreased productivity, and increased treatment costs (Özcan and Gürçay, 2000; Uzal et al., 2003; Greco et al., 2005). A conclusive diagnosis was not only based exclusively on toxin detection but also accompanied by pathological as well as microbiological findings (Uzal and Songer, 2008). Three clinical forms had been identified; the first form associated with *C. perfringens* type A infection (yellow lamb disease or hemolytic disease of sheep) and characterized by acute onset of depression, pale mucus membrane, dyspnea, and severe abdominal pain, then death on the same of onset of illness or some animals may die within few days later. The second form associated with *C. perfringens* type B infection (lamb dysentery and/or hemorrhagic enteritis) sudden death is the main feature of this form. Sometimes, symptoms of depression, abdominal pain, and severe bloody to greenish diarrhea were noticed. The last observed form was associated with *C. perfringens* type D infection (pulpy kidney disease) which begins with the sudden death of few cases, and the number of died animal markedly increased within a week or more from the first record of death. In addition, many signs were observed such as diarrhea, inappetence, depression ended by collapse and death. Noteworthy, some animals suffered from progressive weakness with the development of nervous signs such as dullness, ataxia, incoordination and convulsive movement of the head with the neck rest laterally on the shoulder. Regarding the necropsy finding, the data obtained showed that the rumen is full of ingesta, severely congested intestine and bloody fluids in the body cavities. While, histopathological examination of tissue samples revealed that, severe necrosis and destruction of intestinal villi, hemorrhages, edema in the intestinal lamina propria with severely congested blood vessels (Figure 2A and B). The liver showed dissociation of hepatic cords, hemorrhages, congestion of central vein, edema under the endothelial lining of hepatic sinusoid with portal edema and coagulative necrosis (Figure 2C and D). The kidneys showed changes in the form of hypercellularity with red blood cells infiltration in Bowman's space, medullary hemorrhages and nephrosis with necrosis of proximal and distal convoluted tubules (Figure 2E, F and G). Malacia, demyelination, perivascular hemorrhages, edema and congestion of both cerebrum and cerebellum (Figure 2H). Previous studies showed variable histopathological signs are quite similar to those presented herein (Greco et al., 2005; Van Immerseel et al., 2010). Complete identification of the isolates had revealed that all the isolates were Gram-positive, spore-forming bacilli, produce double zone of hemolysis on sheep blood agar with an incidence rate of 41, 12 and 59% of collected soil, apparently healthy and diseased sheep samples, respectively. Conversely, Habashy el al. (2009) failed to recover any *C. perfringens* isolates from apparently healthy sheep with no data about the role of soil as a potential source of infection. This can be explained as, Habashy et al. (2009) mainly focused on bacteriological isolation and characterization of clostridia with no further molecular identification of toxin type. In addition, their study was conducted in another province; and this strongly recommended further studies to evaluate the regional distribution of clostridial species in Egypt. Conversely, this result is lowered in comparison with that obtained by Mahmoud (1991) and Osman (1993), who successfully isolated *C. perfringens* from 66.5%, 17.74% of samples collected from healthy sheep. While, in case of diseased sheep, the data presented in this study is lower in comparison with that obtained by Abd El-Moez et al. (2014), who showed that, *C. perfringens* was recovered from 77.8% of diseased sheep with no data regarding apparently healthy and soil. The existence of clostridia spores in soils plus in apparently healthy sheep can produce sporadic diseases episodes that are accountable for massive economic losses in animal production through ingestion of the organism and then toxin release (Gamboa et al., 2005; Diego et al., 2012). Therefore, the data presented in this study is considered significant to emphasize the role of soil and apparently healthy sheep in the incidence of *C. perfringens*–induced sheep enterotoxaemia. Hence, further studies are requisite to authenticate the molecular association among *C. perfringens* isolated from soil and

apparently healthy sheep in one hand and diseased sheep in the other to trace the source of infection. Based on the result of multiplex PCR, *C. perfringens* type A was the predominant followed by *C. perfringens* type D in either isolate of diseased sheep, apparently healthy sheep, and soil. This is consistent with the findings of Gerco et al. (2015), who showed that *C. perfringens* type A and D are the predominant causes of predominant causes of enterotoxaemia in very young lambs and kids in southern Italy. Similarly, Abd El-Moez et al. (2014) showed that *C. perfringens* type A is the predominated type isolated from humans and animals. In contrast, previous studies showed that the main cause of sheep dysentery in UK, South Africa, and Greece was *C. perfringens* type B (Gkiourtzidis et al., 2001; Bueschel et al., 2003). Noteworthy, the finding that no *C. perfringens* type E strains were identified strongly advocate that *C. perfringens* type E is rare in lambs and kids (Greco et al., 2005). *C. perfringens* in general are associated with several forms of enteric diseases including fatal enterotoxemia in animals. *C. perfringens* type A is the main causative agent of gas gangrene (myonecrosis) and diarrhea (Hatheway, 1990), while type B and type D are the predominant causes of fatal enterotoxemia in domestic animals (Yamagishi et al., 1997). *C. perfringens* type A is one of the major toxin producers among clostridia species; however, alpha is representing the main toxin type for this species (Popoff and Bouvet, 2013). This species is commonly isolated from environment and intestine of both healthy and diseased animals while other toxin types are less frequently isolated from intestinal cultures, and sometimes can be isolated from the environment in areas where the clostridial disease is present (Songer, 1996). The precise pathogenesis of enteritis induced via clostridial toxin production still needs more investigation (Timbermont et al., 2011) and most probably the production of the alpha toxin is the key element. *C. perfringens* type B and type D produce are the main producers of epsilon toxin, the infection with this subspecies is manifested via the production of epsilon toxin as a result of overfeeding, the toxin abundantly synthesized, then absorbed into circulation as a result of increased intestinal permeability, toxin then spread to various internal organs including brain, kidney, and lung causing edema, neurological disorders, and ultimately mortal effect often ends with sudden death (Finnie, 2003). Similarly, the data provided showed that the main clinical feature of the sheep infected with this subspecies is sudden death. Collectively, the data obtained herein accentuated the significance of multiplex PCR in combination with necropsy, histopathological findings and bacteriological isolation and identification to verify the infection with *C. Perfringens*. Furthermore, the data emphasize the role of soil and apparently healthy sheep in the pathogenesis of clostridial infection as a potential source of infection for the second episode, however, the genetic relationship between the various strains isolated need further investigation. Finally, the high positivity rate to *C. perfringens* type A toxins remarkably counsel counting of this strain in vaccine schedule in order to validate the ample guard to avert the disease in animals.

Conflict of Interests

The authors have not declared any conflict of interests.

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