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NOVEL POTENTIAL PROBIOTICS FROM GUT MICROBIOTA OF HONEYBEES (*APIS MELLIFERA*) IN CLOVER FEEDING SEASON IN EGYPT.

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Abstract

A total of 128 microbial isolates obtained from the gut of honey bees in different life stages in 2017 clover feeding season in Egypt was assessed for in vitro antagonistic activity against human pathogens. 52 out of 128 isolates have an antagonistic effect against one or more pathogens. 5 isolates out of 52 showed a high potential to act as probiotics. They were identified by molecular means as *Wickerhamomyces anomalus, Lachancea thermotolerans, Zygosaccharomyces mellis, Bacillus licheniformis* and *Paenibacillus polymyxa*. The antagonistic mechanisms were analyzed using cylinder diffusion assay and transmission electron microscopy. They tolerate pH up to 1.5 and bile salt conc. up to 3% after 3 hours of incubation. GC/MS analysis revealed the presence of Heptadecane, Palmitic acid, Dodemorph, Paraldehyde, Octadecnoic and Fenoprofen which may inhibit the growth of pathogenic microbes. Although it seems unconventional to use yeast strains and non-lactic acid bacteria as probiotics, these five isolates have a high potential to be used as probiotics for animals and humans.

***Key words*:** Bacterial probiotics; *Lachancea thermotolerans;* Untraditional probiotics; *Wickerhamomyces anomalus;* yeast probiotics; *Zygosaccharomyces mellis*

Introduction

The honey bee microbiome has been studied extensively in the last decade; it was found that the digestive tract of the honey bees contains a large microbial community with special characteristics (Kwong *et al*., 2016). Part of this community is the probiotics which confer a health benefits for both honeybees and humans. Probiotics are known to be non hemolytic, antibiotic resistant microorganisms which can robustly colonize the intestine and tolerate extreme gastric acidity and bile salt effects (Saad *et al*., 2013 and Vijaya *et al*., 2015). Yeasts are also proved to be helpful as probiotics (Chi *et al.,* 2010 and Liu *et al.,* 2013) they can decrease pH and produce variable antimicrobials such as mycocins (Suzuki *et al*., 2001 and Schmitt *et al*., 2006). Yeast probiotics have many advantages over bacterial probiotics. Yeast cell wall is made of chitin or mannose not peptidoglycan or lipoploysaccharides. It grows on acidic pH while bacteria grow normally at neutral conditions. It is also resistant to most antibiotics and does not have the possibility of horizontal gene transfer, so it is genetically stable more than bacteria. (Czerucka *et al.,* 2007). Several researchers proved the efficiency of the use of unusual probiotic bacteria. *Paenibacillus* species was first claimed by Naghmouchi *et al*., (2013) as a potential probiotic. Thereafter *B. amyloliquefaciens* and *B. subtilis* were reported by Du *et al*., (2018) to improve growth performance in weak animals by enhancing rumen, intestine and hormone functions. Lee *et al.,* (2017) recommended the probiotic strain *B. amyloliquefaciens* LN for applications in human diet without any hemolytic properties or food toxicity.

This study was planned to screen the honeybee gut microbiota from the Egyptian honeybees for selecting potential probiotic strains in order to be applied in animal feed recipes after passing the recommended safety and quality insurance tests.

Materials and methods

Honeybees sampling and microbial isolation

This part of the study was conducted on honeybee

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colonies from the apiary of the Faculty of Agriculture, Ain Shams University during the clover feeding season 2017. Samples of worker honeybees and immature stages (larvae and pupae) were collected from hybrid honeybee strains [hybrid of *Apis mellifera carnica* (Craniolian bees) and *A. mellifera ligustica* (Italian bees)] for microbial isolation. Samples were collected in 20-ml tubes containing sterilized physiological saline (0.9% NaCl, 0.1% tween 80 and 0.1% peptone). Adult honey bee workers were dissected and three parts of the elementary canal were used. The first part, *i.e.,* honey stomachs (the fore gut). The second *i.e.,* stomach (the mid gut). The third *i.e.*, rectum (the hind gut). Meanwhile, selected immature stages (larvae and pupae) were grinded in Petri dishes with 1 ml saline for preparation of isolation. (Cakici *et al*., 2014). For microbial isolation, the adult honeybees workers were frozen at -20°C for 20 minutes. The surfaces of tested adult workers were sterilized by 70% ethanol for 5 minutes. The surface of larvae and pupa were also sterilized by the same technique followed by washing them by water for 3 times. The larvae and pupae were aseptically grinded by glass rod (Vojvodic *et al*., 2013). Honey stomach, stomach (ventricles) and rectum of adult honeybee workers were immediately transferred into two different media which are osmophilic agar (Downes *et al.,* 2001) and glucose agar (Waisbren *et al.,* 1951) incubated at 30°C for 24-48 hrs. to obtain pure isolates.

Antimicrobial activity

Isolates obtained from honey bee samples were tested for their antagonistic activities against four reference strains of human bacterial pathogens, Methicillin Resistant *Staphylococcus aureus* (*MRSA*) ATCC 43300, *E. coli* O157 :H7 ATCC 700728, *Pseudomonas aeroginosa* ATCC 9027 and *Salmonella enteric sub sp. Enteric serovar typhimurium* ATCC 14028) and one yeast pathogen *Candida albicans* ATCC 10231. The cylinder diffusion method was used according to (Rashad *et al.,* 2015) on the respective medium for each microorganism. All experiments were done in triplicates and the antimicrobial activities were expressed as the diameter of growth inhibition zones (cm).

Haemolytic activity

Isolates (24 hrs. old) were streaked on Columbia-agar medium containing 5% human blood and incubated at 37°C for 24 hrs. The non-haemolytic isolates were selected (Yigilca *et al.*, 2017).

Tolerance to low pH

Four different pH (pH 7.4, 3, 2 and 1.5) phosphate buffer saline solutions were prepared. The phosphate buffer composition was 1.44 g/L Na_2HPO_4, 8g/L NaCl, 0.2g/L KCl and 0.24 g/L $K_2H_2PO_4$. Drop plate technique was used to determine the tolerance to low pH (Sanders, 2012).

Tolerance to high bile salt concentration

Four different bile concentrations from the two media (glucose agar or osmophilic agar) were prepared (0%, 0.5%, 1% and 3%). Drop plate technique was used to determine the tolerance to high bile salt concentration (Sanders, 2012).

Antibiotic Resistance of the probiotic strains

The five pioneer antagonistic strains isolates examined for their resistance against 7 antibiotics namely, Chloramphenicol (C) 30 mcg, Trimethromycin (E) 15 mcg, Trimethoprim/Sulphamethoxazole (SXT) 1.25\23.75 mcg, Carbenicillin (PY) 100 mcg, Gentamicin (CN) 10 mcg, Cefazolin (CZ) 30 mcg and Amikacin (AK) 30 mcg. Antibiotic disk diffusion method was applied according to Wilkins *et al.,* (1972) and Turhan (2011) on the respective medium for each microorganism where the diameter of growth inhibition zone were measured in cm.

Statistical analysis

All experiments were done in triplicates. The statistical analysis was done according to (Gomez *et al.,* 1984). All data obtained from antagonistic and antibiotic experiments were statistically analyzed and L.S.D. values at the 0.01 level of significance were used for comparison between means.

Identification of isolates

For each isolate, Gram staining and colony morphology were tested. For molecular identification of pure isolates, total genomic DNA was extracted according to Devi *et al*., (2015) and DNA was kept at -20°C until use.

A-16s rRNA gene amplification of bacterial isolates : The PCR amplification of 16S rRNA gene using genomic DNA was performed using oligonucleotide primers (U8-27(F): 5'-AGA GTT TGA TCM TGG CTC AG-3' and R149-1514(R): 5'-CTA CGGY TAC CTT GTT ACG AC-3'). The reaction volume was 25 µL including 11.125 µl QH_2O, 5.0 µl 5x GoTaqFlexi, 2.5 µl 2 mM dNTPs, 2.5 µl 25 mM $MgCl_2$, 1.25 µl DMSO = 5%, 0.75 µl forward primer (0.3 µM), 0.75 µl reverse primer (0.3 µM), 0.125 µL (0.625U) Taq DNA polymerase. PCR conditions wereas follows [5 min at 95°C for the initial denaturation of template DNA, 30 amplification cycles (1min at 94°C, 1 min at 56°C, 2 min at 72°C) and a 10 min at 72°C for the final extension]. (Heuer *et al*., 2009). Estimated PCR product size was 1506 bp.

B- ITS region gene amplification for yeast isolates : Partial sequencing of the ITS gene was used to identify the yeast isolates .The primers for amplification of the ITS region were as follows TW81(F): 5'-GTT TCC GTA GGT GAA CCT GC-3' and AB28(R): 5'-ATA TGC TTA AGT TCA GCG GGT-3'. The reactions were 20 µL including: 8.6 µl QH_2O; 3µl (5x) buffer GoTaqFlexi, 2 µl (2 mM) dNTPs, 2 µl MgCl2 (2.5 mM), 1.2 µl DMSO (5%), 1 µl forward primer (25 Pico mole), 1 µl reverse primer (25 Pico mole), 0.125 µL (0.625U) Go Taq DNA polymerase. PCR conditions were as follows [5 min at 95°C for the initial denaturation of template DNA, 35 amplification cycles (1min at 94°C, 1min at 58°C, 2 min at 72°C) and 10 min at 72°C for the final extension]. The estimated PCR product size is 800-900 bp(Curran *et al*., 1994). The reaction was carried out using thermo cycler (Bio Rad ThermoT100). PCR products were subjected to clean up, then separated on 2.0% agarose gel, stained with ethidium bromide, and viewed under UV light, then sequencing of the amplified DNA fragments was performed by Macrogene, Inc., China. Comparison of the 16S rDNA gene sequences and ITS sequences with entries in the updated GenBank database was conducted using the NCBI BLAST program.

Gel electrophoresis

Electrophoresis of PCR-amplified products is performed in 1.5% agarose gels (low melting) for 1.5 h. at 7.0 V/cm2 (Sambrook *et al.*, 2001). PCR products are stained with 0.5 g/ml of ethidium bromide and visualized with 305 nm ultraviolet light.

Phylogenetic analysis

The Evolutionary analyses is carried out adopting the MEGA5 soft ware. (Tamura *et al.,* 2011). The evolutionary history is inferred by using the Neighbor-Joining method (Saitou *et al.,* 1987). The evolutionary distances are calculated using the maximum composite likelihood according to Tamura *et al*., (2004).

Biofilm formation

Microtiter Dish Biofilm Formation Assay was used according to (O'Toole, 2011).

Colon anticancer activity

Neutral red uptake method was applied for determination of cell viability and cytotoxicity where the cell survival rate was measured using the colon cancer cell line ($CaCO_2$). A liquid culture was incubated for 48 hrs. before experiment. The supernatant was filter-sterilized by 0.22 µm cellulose acetate filter, diluted to 40% and applied to the cell line (Repetto *et al*., 2008).

Transmission electron microscope imaging

Negative staining for cells was done by staining using sodium phosphor tungstate. (Scarff *et al*., 2018). The sample was examined microscopically by Transmission electron microscope JEOL (JEM-1400 TEM).

Chromatographic analysis using GC–MS

The ethyl acetate extracts of crude cultures are analyzed using GC–MS and performed by Agilent Technologies 7890B GC Systems combined with 5977A Mass Selective Detector. Capillary column is used (HP-5MS Capillary, 30.0 m × 0.25 mm ID × 0.25 µm film) using helium as the carrier gas at a flaw rate of 1.8 ml/min with 1 µl injection. The sample is analyzed with the column held initially for 3 min at 40°C after injection, then the temperature is raised to 300°C with a 20°C/min heating ramp, with a 3.0 min hold. Injection is carried out in split less mode at 300°C. MS scan range (*m*/*z*): 30–500 atomic mass units (AMU) under electron impact (EI) ionization (70 eV).

Results and discussion

Molecular Identification of isolates

The DNA sequences of isolated yeasts and bacteria were compared with the data available in NCBI by using BLAST to estimate the phylogenetic relationships of the microbes. Fig. 1 illustrates the PCR product and fig. 2 illustrates the phylogenetic analysis tree of the BLAST results. In fig. 1 the first 3 lanes from the left are for PCR product of yeast isolates which are *Wickerhamomyces anomalus, Lachancea thermotolerans and Zygosaccharomyces mellis*. The marker appears in lane 4 (250, 500, 750 and 1000 bp). The last 2 lanes in the right after the marker are for PCR

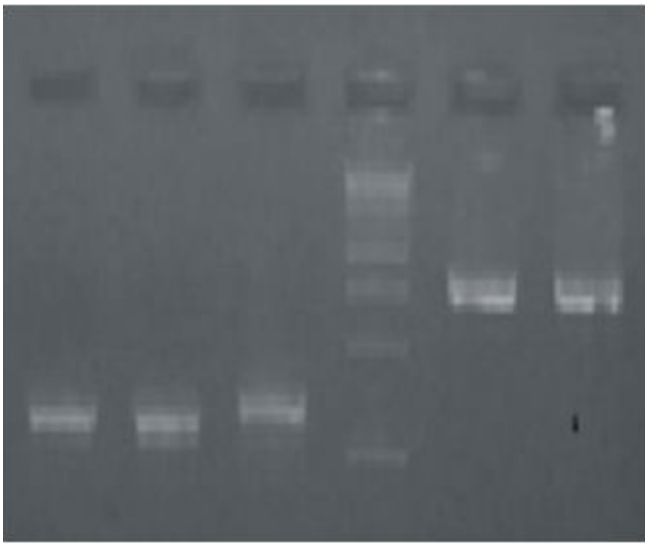

Fig. 1: Gel electrophoresis of PCR product of 5 isolates

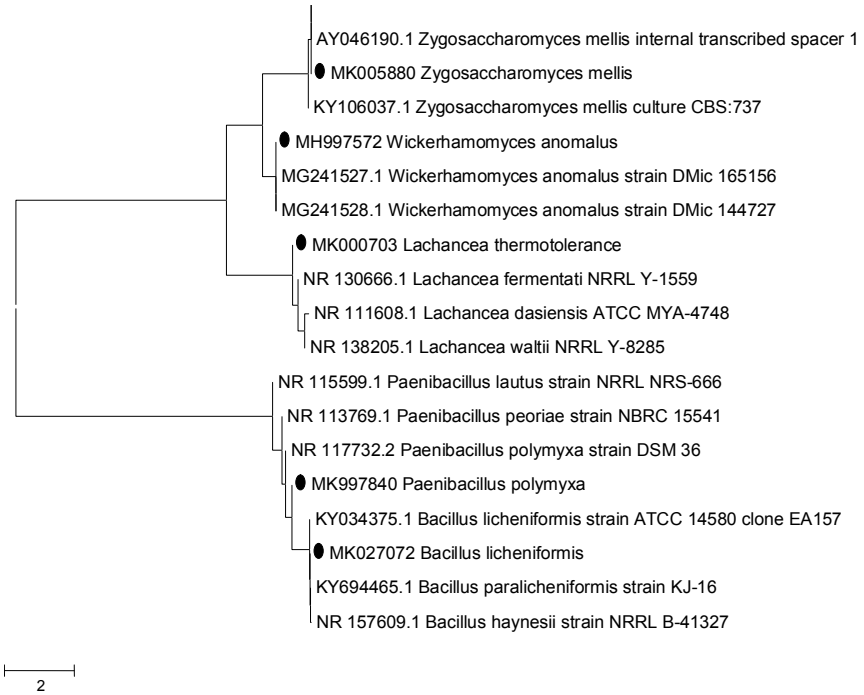

Fig. 2: Phylogenetic tree . The black dots indicate the authors strains compared by strains from NCBI database strain using MEGA5 software

product of *Bacillus licheniformis and Paenibacillus polymyxa,* consequently. The best five microbial isolates were identified, submitted to and deposited at GenBank under the following accession numbers: *Wickerhamomyces anomalus* (MH997572), *Lachancea thermotolerans* (MK000703), *Zygosaccharomyces mellis* (MK005880), *Bacillus licheniformis* (MK027072) and *Paenibacillus polymyxa* (MH997840).

Antimicrobial activity experiment

One hundred and twenty eight isolates were obtained from the clover season from different life stages of honey bees. Screening for the isolates that have antagonistic effect was done. Fifty two isolates have an antagonistic effect against one or more pathogens. Five isolates out of fifty two showed a high potential to act as probiotics. The results presented in table 1 shows the antimicrobial activity of each of the 5 identified isolates. The first isolated strain *Wickerhamomyces anomalus* (OLC350) that was more active against *E. coli* O:157:H7 and *Salmonella typhimuruim* with 3.2 and 2.6 cm inhibition zones, respectively. *Wickerhamomyces anomalus* is known to be able to reduce *Enterobacteriaceae* count and confer a nutritive value by increasing the protein content of the dietary formula (Olstorpe *et al*., 2010). It is also well-known for the production of Exo-β-1, 3glucanases which was reported for its ability to break down the bacterial cell wall component β-1, 3glucan and this explain its mode of action (Serena, 2015). This might explain its antagonistic effect against *E. coli* O157:H7 and *Salmonella typhimuruim.* The second identified strain is *L. thermotolerans* (ORC348) was more active against *E. coli* O157:H7 and *Salmonella typhimuruim* with 2.7 and 3.0 cm inhibition zones, respectively. This bacterium is characterized by their glucophilic character (Hranilovic *et al.,* 2018) and can produces up to 9.6 g/L lactic acid in the growth medium which significantly reduces the pH of the culture and inhibit growth of other bacteria (Kapsopoulou *et al.,* 2005). The third isolate identified as *Z. mellis* (OPC155) was more active against MRSA, *Staphylococcus aureus*, *E.coli* O157:H7, *Salmonella typhimuruim and P.aeruginosa* producing inhibition zones with 2.5, 1.63, 2.5, 2 and 1.3 cm diameter, respectively. The genus *Zygosaccharomyces* spp. produces high ethanol and acetoin as well as antimicrobial peptides like Lactoferricin (Escott *et al.*, 2018) which may explain the mode of its antimicrobial action. The fourth isolated strain is *Bacillus*

Table1 : Antimicrobial activity results of the five pioneer strains

Identified isolates	Code	*Pathogenic strains*					
		MRSA *aureus*	*S.* O:157	*E.coli typhimurium*	*S. aeroginosa*	*P. albicans*	*C.*
		Inhibition zones (cm)					
Wickerhamomyces anomalus	OLC 350	0	0	3.3±0	2.6±0.06	0	0
Lachancea thermotolerans	ORC 348	0	0	2.7±0.15	3±0	0	0
Zygosaccharomyces mellis	OPC 155	2.5±0	1.63± 0.05	2.5±0	2±0.06	1.3±0.06	0
Bacillus licheniformis	GLC 226	0	0	0	0	2.7±0	4±0
Paenibacillus polymyxa	GPC 228	0	0	1.4±0.23	2±0	0	0
* L.S.D. Value =0.2919							

Table 2: Tolerance results of five pioneer strains to Low PH and high Bile salt concentration

Identified isolates	Code	Time (h)	pH tolerance	Bile salt tolerance
Wickerhamomyces anomalus	OLC 350	1	up to 1.5	up to 3%
		2	up to 1.5	up to 3%
		3	up to 2	up to 3%
Lachancea thermotolerans	ORC 348	1	up to1.5	up to 3%
		2	up to1.5	up to 3%
		3	up to1.5	up to 3%
Zygosaccharomyces mellis	OPC 155	1	up to 2	up to 3%
		2	up to 2	up to 3%
		3	up to 7.4	up to 3%
Bacillus licheniformis	GLC 226	1	up to 1.5	up to 3%
		2	up to 3	up to 3%
		3	up to 3	up to 3%
Paenibacillus polymyxa	GPC 228	1	up to 1.5	up to 3%
		2	up to 1.5	up to 3%
		3	up to 1.5	up to 3%

licheniformis (GLC 226) was more active only against *P. areuginosa* and *Candida albicans* with 2.7 and 4 cm inhibition zones, respectively. It produces bacitracin which is a thermostable antibiotic that is resistant to enzymatic degradation, making it very effective in the gut. This in vivo antibiotic also helps the function of further gut-derived antibiotic named subtilisin. Additionally, *B. licheniformis* produces a unchangeable protease enzyme elementary for digesting and assimilating proteins in the body. The fifth strain is *Paenibacillus polymyxa* (GPC 228) was more active against *E.coli* O157:H7 and *Salmonella typhimuruim with* 1.4 and 2.0 cm inhibition zones, respectively, *which* was *proved to* produce polyxin that inhibited growth of a wide array of Gram positive and Gram-negative bacteria, including food-borne pathogens. It has Bacteriocin-like characteristics such as proteinaceous nature, insensitivity to organic solvents and chelators, uniformity to heat (up to 10 min at 90°C) and acidity but alternation in alkaline conditions were observed (Piuri *et al.*, 1998).

pH and bile salt tolerance

Table 3: The results of anticancer activity of the five pioneer isolates and their isolation source

Identified isolates and their code	Source of isolation	Activity against Colon cancer (Viability % at concentration 40%(V/V)
Wickerhamomyces anomalus (OLC 350)	Larvae of Italian workers in clover season	13.5
Lachancea thermotolerans (ORC 348)	Rectum of Italian worker in clover season	10.6
Zygosaccharomyces mellis (OPC 155)	Pupa of Italian drone	0
Bacillus licheniformis (GLC 226)	Larva of workers in clover season	93.5
Paenibacillus polymyxa (GPC 228)	Pupa of workers from clover season	83

Table 4: Antibiotic resistance (inhibition zone diameters cm) by the 5 pioneer strains.

Identified isolatesand its code	E	AK	CN	C	SXT	PY	CZ
Wickerhamomycesanomalus	0	0	0	0	0	0	0
Lachanceathermotolerans	0	0	0	0	0	0	0
Zygosaccharomycesmellis	0	0	0	0	0	0	0
Bacilluslicheniformis	0.6±0	3±0.06	2.1±0.15	3.6±0.1	0	2±0.1	2.7±0.12
Paenibacilluspolymyxa	3±0.34	3.5±0.06	2.4±0.3	4.8±0.29	4±0.06	3.2±0.06	3.2±0.2
*L.S.D. value = 0.2799							

C:Chloramphenicol, E:Trimethromycin, SXT:Trimethoprim/ Sulphamethoxazole, PY: Carbenicillin, CN: Gentamicin CZ: and AK: Amikacin.

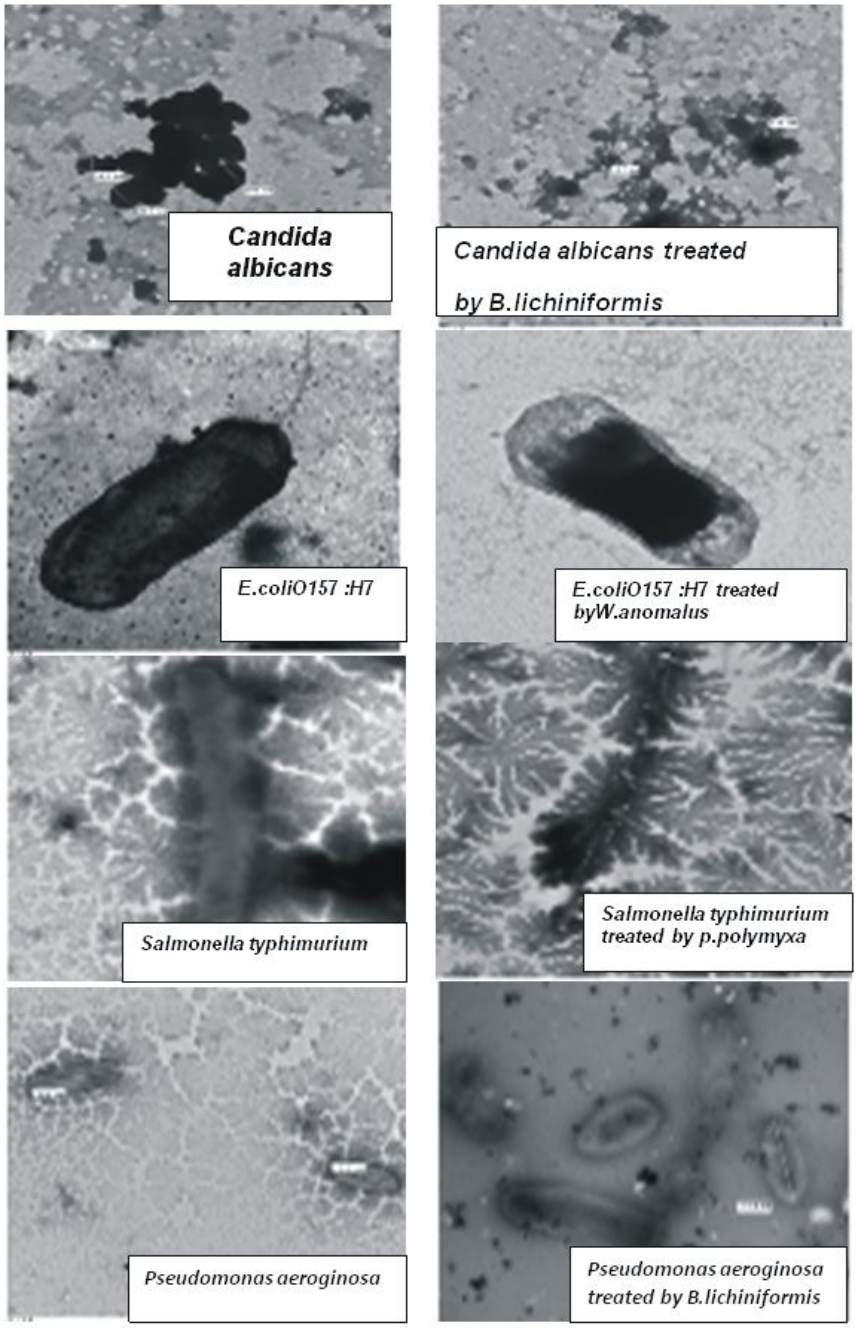

Fig. 3: TEM images of control pathogens and treated with extracts of pioneer strains.

Five isolates out of the examined 52 showed high tolerance to low pH and high bile salt concentration. The principle is if the bacteria could tolerate such conditions will be able to grow again and it probably have a mechanisms of tolerance to such harsh conditions. It's important for the strain to tolerate such conditions in order to be effective probiotics. (Table 2).

Blood hemolysis

10 out of the examined 52 antagonistic isolates are not hemolytic. 5 Out of these 10 non hemolytic antagonistic isolates were resistant to pH and Bile and thus selected for molecular identification and used in further studies.

Antibiotic Resistance by the probiotic strains

Table 4 shows that all the examined yeasts exhibited no inhibition zones on their medium containing the examined antibiotics at all concentrations. The use of antibiotic resistant yeast strains as probiotics is preferable due to their antibiotic resistance capabilities might be attributed to their eukaryotic nature and cell wall structure. Growth inhibition is expressed as the diameters of the inhibition zone (Cm) (Czerucka *et al*., 2007). On the other hand the bacterial strains were sensitive to all the examined antibiotics showing zone diameters ranged between 0.6 and 4.8 cm except with *Bacillus lichiniformis* which grown on the medium containing the antibiotic trimethoprim without any growth inhibition.

Transmision electron microscope (TEM)

As shown in fig. 3, all control samples images illustrate normal and smooth cell morphology. The treated *Candida albicans* image (treated by *Bacillus licheniformis* filtrated extract) showed severe destruction and disturbed cell morphology. The TEM image of treated *E.coli O 157:H7* cells (treated by *Wickerhamomyces anomalus* filtrated extract) showed severe alteration of cell morphology, collapse of bacterial cells and shrinking of internal content. TEM image of *Salmonella typhimurium (treated by P. polymyxa filtrated extract)* showed severe alteration of cell morphology and shrinking of internal content. The TEM image of *Pseudomonas aeroginosa* cells (treated by *Bacillus Lichenformis* filtrated extract) showed shrinking of internal content.

Anticancer activity

As shown in table 3. The three yeast strains *i.e. W. anomalus*, *L. thermotolerans, Z. mellis* showed high reduction in the colon cancer cell line viability. A promising finding is in accordance with those reported

Table 5: Results of GC MS analysis showing the most active components produced by the five pioneer strains

Compound	Strain	Biological activities
Heptadecane	*Bacillus licheniformis*	Antioxidant, anticancer (Dae Hyun Kim *et al.* , 2013)
Pyrrolo [1, 2-a] pyrazine-1, 4-dione, hexahydro-3-(2-methylpropyl)-	*Paenibacillus polymyxa*	One of the quorum sensing compounds in bacteria (pubchem)
hexadecanoic acid (Palmitic acid)	*Paenibacillus polymyxa* *Lachancea thermotolerance*	In foods as a Flavoring Agent, lubricant, binder, &defoaming agent(pubchem)
Dodemorph	*Paenibacillus polymyxa*	Fungicide
Paraldehyde	*Lachancea thermotolerance*	management of acute, including prolonged, tonic–clonic convulsions.(A G Rowland, 2009)
Octadecanoic acid (Stearic Acid)	*Lachancea thermotolerance*	used in a *variety* of cosmetics and personal care products, as a fragrance ingredient, surfactant and emulsifier
Benzoic acid	*Wickerhamomyces anomalus* *Zygosaccharomyces mellis*	fungi static compound that is widely used as a food preservative (pubchem)
n-Hexadecanoicacid(Palmitic acid)	*Wickerhamomyces anomalus*	used to produce soaps, cosmetics (pubchem)
Fenoprofen	*Wickerhamomyces anomalus*	Antiinflammatory and ant rheumatic properties. (pubchem)
2,4-Di-tert-butylphenol	*Zygosaccharomyces mellis*	Antioxidant (pubchem)
Pyrrolo[2,3-b]indole, 1,2,3,3a,8, 8a-hexahydro-5-methoxy-3a, 8-dimethyl-,	*Zygosaccharomyces mellis*	useful as memory-enhancing and analgesic agents. (pubchem)

by Thirabunyanon *et al.,* (2013) who indicated that three probiotic microorganisms *Lactobacillus salivarius*, *Pediococcus pentosaceus* and *Enterococcus faecium* exhibited anti proliferative properties to colon cancer cells.

GC-MS analysis results

The GC mass analysis reveal the most active components from the five pioneer probiotic strains presented in table 5 with a reference to its bioactivity or importance**.**

Conclusion

This work suggests the possibility of using the isolated yeast strains as probiotics from the local Egyptian environment in addition to highlighting other novel bacterial strains that were not recognized before as probiotics. Thus, revealing new functions of known yeasts and bacteria.

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