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Probiotic Potential and Virulence Traits of Bacillus and Lactobacillus Species Isolated from Local Honey Sample in Iran

Niloofar Razmgah[1], Naheed Mojgani[2*] and Mohammad Amir Karimi Torshizi[3]
[1, 3] (Department of Poultry Science, Faculty of Agriculture, Tarbiat Modares University, Tehran)
[2](Department of Biotechnology, Razi Vaccine and Serum Research Institute, karaj- Agriculture Research, Education and Extension Organization, Tehran, IR Iran)

Abstract: *The probiotic potential of bacterial species isolated from local honey samples were analyzed by microbiological, biochemical and molecular methods. A total of 17 Gram positive bacterial isolates were recovered from local honey samples and tested for their hemolysin, coagulase, DNase, Lipase, Lecithinase and Gelatinase producing abilities, respectively. The isolates (5) lacking the tested virulence phenotypes were selected for identification by 16S rRNA sequencing. The probiotic characteristics of the isolates was determined by testing their resistance to acid, bile, antimicrobial spectrum against pathogens and antibiotic sensitivity profile. Furthermore, the identified bacillus species were screened for the presence of virulence genes including Enterotoxin-T (bceT), cytotoxin K (cytk), hemolysin BL (hblA, hblC, hblD) and non-hemolytic enterotoxin gene (nhe). All results were analyzed statistically. According to results, Bacillus megaterium TA008 and Lactobacillus acidophilus RNL26 showed significantly high resistance to acidic pH values and high bile salt concentrations ($p<0.05$). Bacillus subtilis TA049 which was unable to tolerate extreme acidic conditions (pH 2.0, 2.5), survived significantly ($p<0.05$) in high bile salts concentrations. All isolates demonstrated antibacterial activity against the pathogens to variable extents. Widest spectrum of action was shown by B.subtilis TA049 which inhibited the growth of Enterococcus faecalis, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi and Staphylococcus aureus. While, B.megaterium TA009 and Brevibacillus brevis TA010 were active only against E.faecalis and S.typhi. Antibiotic sensitivity profile of the isolates varied with B.megaterium TA008 being sensitive to all the tested antibiotics ($p<0.05$) while L.acidophilus RNL26 was resistant to nalidixic acid and vancomycin. B.megaterium TA008 and B.subtilis TA049 were devoid of the tested virulence genes while, B.megaterium TA009 harbored the hemolysin genes (hblA, hblC) and B.brevis TA010carried the cytotoxin gene (cytK).*

Keywords*: Bacillus subtilis, B.megaterium, Brevibacillus brevis, Lactobacillus acidophilus, Honey samples, Probiotic bacteria.*

I. Introduction

Since decades' people have being using honey for medicinal purpose including treatment of burns, wounds, ulcers, sore eyes etc. Furthermore, honey has also been employed to shorten the duration of diarrhea in patients with bactericidal gastro-enteritis due to bacterial infection (Haffejee and Moosa, 1985). The therapeutic effect of honey has been attributed to the natural microbiota of the honeybee gut. According to reports, honey is non-sterile with a natural bacterial flora ranging between 0 and 5000 CFU/g, composed mostly of Gram-positive aerobic spore forming bacteria, such as Bacillus spp. which accounts for an average of 60% of bacteria recovered, depending on the amount of processing of the honey (Snowdon and Cliver, 1996).

The natural bacterial flora of honey has been known to play an essential role in stimulating immune response and hence increasing the resistance to pathogens (Evans and Lopez, 2004). Aween and his co-workers (2010), reported a number of honey bacteria to be active against a number of gram positive and gram negative pathogenic and spoilage bacteria (Aween., *et al* 2010). A number of bacillus species has been the dominant species found in honey samples accounting for approximately 60% of the total bacteria recovered (Snowdon and Clever, 1996). While, Bahiru., *et al* (2006) showed that besides Lactic Acid Bacteria (LAB), honey also contains yeast and low number of spores. In a study conducted by Hosny and his co-workers (2009), presence of *B. butyricum, B.subtilis, Enterococcus faecium, L.acidophilus, L.casei, L. plantarum, Lactococcus lacits, Lact. Cremoris* and *Micrococcus luteus*, in three different Egyptian honey sample has been recorded.

In last few decades, the importance of honey as a reservoir for probiotic bacteria has been evaluated and a number of bacillus and Lactic Acid bacteria (LAB) isolated from this sweet and viscous liquid has gained attention for their beneficial health effects (Rokop et al 2015). The origin of these bacteria in honey samples might not only be the insect itself but the handling of the final product by the beekeeper may also be responsible for introducing microorganisms. This means that organisms found in the environment around honey (air, dust, flowers, soil) are likely to be identified in honey (primary contamination).

Today probiotics are commonly used in health promoting functional foods for humans, and quite well known for their therapeutic, prophylactic effects in animal production and human health (Abdel karim *et al.,*

2012). A number of probiotic bacteria originating from honeybee microbiota are potential probiotic candidates. Generally, bacteria claimed as a probiotic should possess the essential properties such as resistance to stressful conditions of stomach and upper intestine where acid and bile are the most detrimental factors effecting growth and viability (Shah, 2001). Moreover, prior to the application of a LAB as a probiotic source in the food and feed industry, one of the pre-requisites for ensuring consumer safety (EFSA, 2008; 2012) is that it should be assessed for its safety properties. In this study we evaluated the probiotic potential and safety of bacillus and lactobacillus species isolated from local honey samples.

II. Material and Methods

2.1 Bacterial isolation, Identification and Cultural conditions

One gram of honey samples homogenized in a water bath at 40ºC, were mixed vigorously with 1 ml of sterile PBS (pH 6.0) to obtain a 50% honey solution. Using sterile glass spreader, 100 microliters of the suspension was spread evenly on Brain Heart infusion (BHI, Merck; Germany), deManRogosa and Sharpe (MRS), and Tryptic Soya (TSA; Sharlau Spain) agar plates in duplicate. All plates were incubated at 35ºC for 24 to 48 hrs under aerobic and anaerobic conditions. Morphologically different colonies were selected and streaked on fresh BHI and MRS agar plates to obtain pure colonies. After overnight incubation at 35ºC the individual colonies were picked and tested for Gram stain, spore stain, motility and catalase activity. The isolates were maintained on semisolid BHI slants at 4˚C and renewed every week for short-term preservation. For long term storage all isolates were preserved in 20% BHI sterile glycerol stock at -70C.

2.2 Phenotypic virulence determinants

The selected isolates were tested for the production of hemolysin, gelatinase, lecithinase, lipase, coagulase and DNAse as described below

- Hemolytic Activity: Aliquots of the cultures were spotted onto plates containing TSA (Oxoid) added to 5% (v/v) defibrinated horse blood and incubated at 37°C for 48 h. Clear halos around the colonies indicated total or β- haemolysis, and green halos around the colonies indicated partial or α-haemolysis. Absence of halos around the colonies was interpreted as no haemolytic activity (γ-haemolysis).
- Gelatinase test: Overnight grown cultures of selected isolates were spotted on plates containing Luria–Bertani agar (LB, Merck, USA) supplemented with 3% (w/v) gelatine and incubated at 37°C for 48 h, followed by incubation at 4°C for 4 h. Opaque halos around the colonies were recorded as positive results.
- Lecithinase test: lecithinase activity was determined by the method described earlier (Van Netten & Kramer, 1992). The plates were observed for halo of precipitation around the colonies which would detect production of phosphaditylcholine hydrolase (insoluble diaglycerol), while clearing of egg-yolk media around the colonies indicated proteolysis–lipolysis activity.
- Lipase test: For lipase production, aliquots of isolates cultures were spotted onto plates containing LB agar supplemented with 0·2% (w/v) $CaCl_2$ and 0·1% (w/v) Tween 80 (Sigma-Aldrich) and incubated at 37°C for 48 h. Opaque halos around the colonies were recorded as positive results.
- Coagulase tests: The isolates were tested for coagulase activity (bound and free coagulase) by means of standard procedures developed for staphylococci. In brief a colony was suspended in 0.5 ml of horse plasma in a sterile screw-capped tube and. The results were read after 4, 8, and 24 hrs for clot formation which would indicate plasma coagulation. The slide procedure was used to determine bound coagulase enzyme (clumping factor) by mixing with a wooden stick a single bacterial colony from a 24 h culture in BHI agar in saline in a clean microscope slide with one drop of horse plasma. In positive cases, a visible clumping of cells appeared within 20 seconds which indicated coagulation of plasma.
- DNAse test: Aliquots of the isolate cultures were spotted onto plates containing DNAse methyl green agar plates (BD, USA) and incubated at 37°C for 48 h. Clear halos around the colonies were recorded as positive results.

2.3 Genotypic identification of the isolates

The selected isolates lacking the phenotypic virulence traits were identified to species level by 16S rRNA gene sequencing. PCR parameters and universal primers targeting the genus level of *Bacillus* spp (bac-F and bac-R) and lactobacilli (7F and 1510R) were used according to the protocols described by Wu, *et al* (2006) and Satokari and his colleagues (2001), respectively. The amplified products were purified using Gene JET PCR purification kit (Thermo Scientific, Lithuania) and sequenced commercially by First BASE laboratories, Malaysia. The obtained sequences were aligned to 16S rRNA gene sequences in the GenBank data base using the BLAST algorithm available at the National Center for Biotechnology Information (NCBI, USA).

2.4 Probiotic Characterizations

2.4 Probiotic Characterizations

The selected isolates identified as *B.megaterium* (TA008 and TA009), *B.brevis* (TA010) *B.subtilis* (TA049) and *L.acidophilus* (RNL26) were evaluated for their *invitro* probiotic properties by the following tests. 2.4.1. Acid and bile tolerance: The acid and bile tolerance of the selected isolates was determined as described earlier (Mojgani et al 2015). The isolates were incubated in acidic environment of pH 2.0, 2.5, 3.0, 4.0, 5.0 and 6.0 and OD at 560 nm was observed hourly for a period of 4 hrs. Percentage survival was calculated at respective time intervals by the below given formula;

$$\textbf{Survival \%} = \frac{\Delta DO_{pH6.5} - \Delta DO_{pHt}}{\Delta DO_{pH6.5}} \quad (1)$$

The percent difference between the variation of optical density (OD) at pH6.5 ($\Delta OD_{pH6.5}$) and the variation of optical density at the tested pH (ΔOD_{pHt}) would give an index of survival of the isolates at the tested pH values.The bile resistance of the isolates at 0.3, 0.7 and 1.0% bile were tested by incubating the freshly grown cultures of the isolates in study in MRS broth supplemented with the said concentrations of the salt. The growth after 6 hrs under said conditions was recorded by reading the OD and calculating the coefficient of inhibition (C_{inh}) by the given formula (Gopal *et al*. 2001);

$$C_{inh} = \frac{\Delta_{T8-T0}\,\textbf{Control} - \Delta_{T8-T0}\,\textbf{Treatment}}{\Delta_{T8-T0}\,\textbf{Control}} \quad (2)$$

Where, Δ_{T8-T0} represents the difference in absorbance at time zero (T0) and after 8 hrs (T8). C_{inh} of less than 0.4 was considered significant for the isolates to be considered as a suitable probiotic candidate. 2.4.2. Antimicrobial Spectrum: The isolates in study were tested for their antimicrobial spectrum by the well diffusion agar assay described earlier by Vaseeharan and Ramasamy (2003). The pathogens used in study were *Escherichia coli, Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella typhi and Staphylococcus aureus*. The mentioned isolates were cultured in blood and brain heart infusion agar, incubated at 37ºC for 18-24 hrs. The antimicrobial activity was recorded as appearance of clear zone around the wells and zone diameter recorded in millimeters (mm).

2.5 Phenotypic Antibiotics resistance profile

During preliminary screening, the phenotypic antibiotic resistance pattern of the isolates in study was determined by disc diffusion assay (Halami *et al.,* 1999). All antibiotic discs used in study {ampicillin 10ug (AM), cephotaxime 30ug (CTX), chloramphenicol 30ug (C), ciprofloxacin 2U (CIP), clindamycin 30ug (CL), erythromycin 15ug (E), gentamycin 10ug (GN), kanamycin 30ug (KAN), nalidixic acid 30ug (NA), rifampicin 5ug (RA), streptomycin 10ug (SE), tetracycline 30ug (TET) and vancomycin 30ug (V)} were purchased from Sigma (St. Louis, Mo., USA). After placing the respective antibiotic discs on the agar plates the plates were incubated at 37°C for 24 hrs to 48 hrs. After incubation, the plates were observed for zone of inhibition around the discs and diameters recorded (millimeters). All tests were performed in duplicates. Inhibition zone diameters were measured inclusive of the diameter of the discs. Results were expressed as sensitive, S (>21mm), intermediate, I (16- 20 mm) and resistant, R (<15 mm) as described by Vikova and his co-workers (2006). In this experiment we analyze the data by SAS to see check the differences between isolated strains.

2.6 Genotypic virulence determinants

The isolated Bacillus species were analyzed for their virulence determinants by subjecting their genomic DNA to a set of virulence primers. Total genomic DNA was extracted by Commercial DNA isolation kit (Fluka, Germany). Table 1 illustrates the primers their sequence, size of expected amplicons and PCR parameters. *B.cereus* RTCC11145 was used as a positive control for the presence of *hbl* and *bceT* enterotoxins, while *L. monocytogenes* was used as negative control in this PCR reaction.

2.7 Statistical Analysis

All data resulted from experiments with 3 replicates were analyzed with SAS with glm procedure at significant level %0.05.

Table1: The primers and PCR parameters used for genotypic characterization of virulence determinants

Virulence genes	Primer sequence	Amplicon size (bp)	PCR parameters	Reference
HblD-N *HblD-C*	AAT CAA GAG CTG TCA CGA AT CAC CAA TTG ACC ATG CTA AT	430bp	1 cycle 95°C, 5min C for 30sec°cycles, 95 30 4C for 30 sec°5 C for 90 sec°72 C, 5 min°cycle 72 1	Hansen and Hendriksen, 2001
BceT-N *BceT-C*	TTA CAT TAC CAG GAC GTG CTT TGT TTG TGA TTG TAA TTC AGG	428bp	1 cycle 94°C, 5min C for 20sec°cycles, 94 35 56C for 20 sec C for 20sec°72 C,5 min°cycle 72 1	Agata et al 1995
HblC-N *HblC-C*	AAT AGG TAC AGA TGG AAC AGG GGC TTT CAT CAG GTC ATA CTC	399bp	1 cycle 94°C, 5min C for 30sec°cycles, 94 36 C for 20 sec°62 C for 1 min°72 C, 5 min°cycle 72 1	Ryan et al 1997
HblA-N *HblA-C*	GCT AAT GTA GTT TCA CCT AGC AAC AAT CAT GCC ACT GCG TGG ACAATA	873bp	1 cycle 95°C, 5min C ,1 min°cycles, 95 30 C ,1min°65 1min ,C°72 min C, 5°cycle 72 1	Ryan et al 1997
CytKF-2 *CytKR-5*	ACA GAT ATC GGT CAA AAT GC CAA GTT ACT TGA CCT GTT GC	421bp	1 cycle 94°C, 5min C, 30sec°cycles, 94 35 C for 45 sec°56 C, 1min°72 min 2,C °cycle 72 1	Ehling-Schulz et al 2006
NheB-F *NheB-R*	AAG CTG CTC TTC GTA TTC TTT GTT GAA ATA AGC TGT	766bp	1 cycle 95°C, 5min C, 30sec°cycles, 95 30 C, 30 sec°52 72C, 1min min C for 2°cycle 72 1	Granum et al 1999

III. Results

A total of 17 gram positive bacteria were recovered from the selected honey samples, of which five were β hemolytic while rest were either α or γ hemolytic. These isolates were further checked for their phenotypic virulence determinants including Gelatinase, coagulase, lecithinase, DNase and Lipase activity. As depicted in Table 2, five isolates appeared to lack all the tested virulent phenotypes. Based on sequences alignment, the isolates were identified as *Bacillus megaterium* (TA008 and TA009), *Brevibacillus brevis* (TA010), *Bacillus subtilis* (TA049) and *Lactobacillus acidophilus* (RNL26), respectively.

Table 2: Microbiological and Biochemical characterization of bacterial isolates from honey samples.

No	Isolates	Gram Reaction	Catalase Reaction	Hemolysis	Lipase Activity	Gelatinase Activity	DNAse	Coagulase Activity	Lecithinase Activity
1	TA001	*G+ve*	+ve	α	-ve	-ve	+ve	+ve	+ve
2	TA002	*G+ve*	+ve	γ	+ve	-ve	+ve	+ve	-ve
3	TA003	*G+ve*	-ve	β	ND	ND	ND	ND	ND
4	TA004	*G+ve*	-ve	β	ND	ND	ND	ND	ND
5	TA005	*G+ve*	-ve	β	ND	ND	ND	ND	ND
6	TA006	*G+ve*	+ve	α	+ve	-ve	-ve	+ve	+ve
7	TA007	*G+ve*	+ve	α	+ve	+ve	-ve	+ve	+ve
8	TA008	*G+ve*	+ve	α	-ve	-ve	-ve	-ve	-ve
9	TA009	*G+ve*	+ve	α	-ve	-ve	-ve	-ve	-ve
10	TA011	*G+ve*	+ve	β	ND	ND	ND	ND	ND
11	TA012	*G+ve*	+ve	β	ND	ND	ND	ND	ND
12	TA014	*G+ve*	+ve	α	-ve	-ve	-ve	-ve	-ve
13	TA049	*G+ve*	+ve	γ	-ve	-ve	+ve	-ve	-ve
14	RNL22	*G+ve*	-ve	γ	-ve	+ve	+ve	-ve	-ve
15	RNL26	*G+ve*	-ve	γ	-ve	-ve	-ve	-ve	-ve
16	RNL27	*G+ve*	-ve	γ	-ve	-ve	-ve	+ve	+ve
17	RNL28	*G+ve*	-ve	γ	-ve	+ve	+ve	-ve	-ve

α hemolysis: partial hemolysis; β: complete hemolysis and γ : no hemolysis; ND: not determined

The identified isolates were tested for their probiotic properties by evaluating their acid and bile resistance, antibacterial spectrum of action against pathogens, antibiotic sensitivity profile and presence of genotypic virulence determinants.

Figure 1, shows the survival ability of the isolated at pH 2.0. According to the obtained results, the two megaterium species showed highest acid tolerance compared to the other isolates in study as they resisted extreme acidic conditions for more than 4 hrs. *B.subtilis* TA049 appeared to be the least resistant strain as it could not tolerate pH values of 2.0 and 3.0.

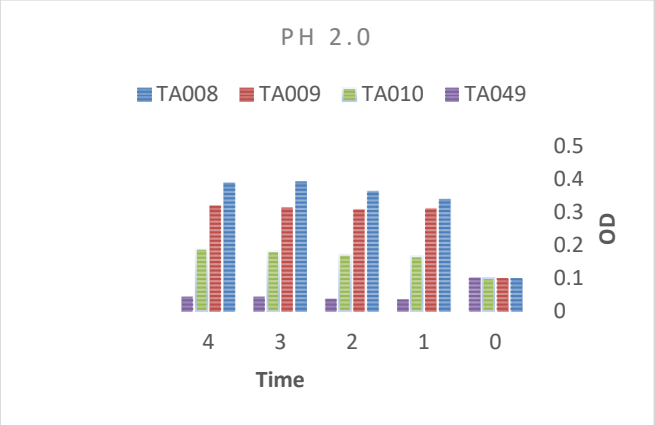

Figure 1: Acid tolerance of the tested isolates at pH 2.0 at different time intervals.

Table 3 shows the bile tolerance of the local honey isolates in the presence of different concentrations of bile salt. Figure 2, depicts the coefficient of inhibition of the isolates in the presence of 1% bile salt after 2, 4 and 6 hrs. Based on our results, *L.acidophilus* RNL26 and *B.subtilis* TA049 were the most tolerant strains under these conditions as they showed the least C_{inh} and alternatively highest growth in the presence of said bile salt concentrations.

Table 3: Coefficient of inhibition of the tested isolates at different bile salt concentrations after

Bacterial isolates	**(ΔT8-T0 Control - ΔT8-T0 (Treatment) / (Δ T8-T0 Control = C_{inh}**		
	0.3% bile	0.7% bile	1.0% bile
TA008	0.18	0.98	0.86
TA009	0.97	0.98	0.97
TA014	1.3	1.1	0.37
TA049	0.78	0.83	0.83
RNL26	0.06	0.10	0.03

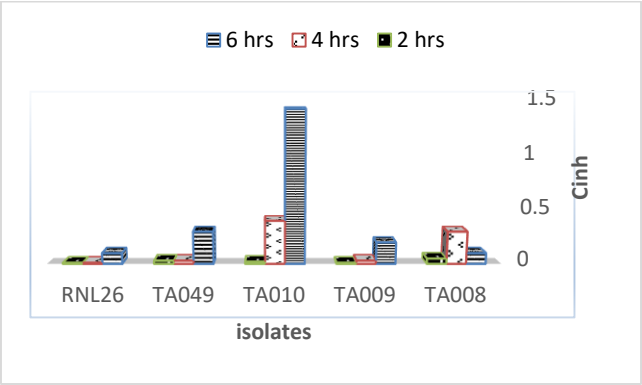

Figure 2: Bile resistance of the test isolates in the presence of 1% bile salt at different time intervals

As shown in Table 4, widest spectrum of inhibition was demonstrated by *B.subtilis* TA049 ($p< 0.05$), followed by *B.megaterium* TA008. The difference in inhibitory spectrum between the isolates was significantly meaningful ($p< 0.05$). None of the isolates were effective against *Pasteurella multocida* and *Klebsiella pneumonia*, while all were effective against *Enterococcus faecalis*.

Table 4: Antimicrobial spectrum of the identified isolates against a variety of pathogenic bacteria

Pathogens	TA008	TA009	TA010	TA0049	RNL26
Streptococcus agalactiae RTCC 2051	-	-	-	+	+
Streptococcus pyogenes ATCC 19615	+	-	-	+	+
Enterococcus faecalis ATCC 51299	+	+	+	+	+
Pasteurella multocida ATCC 43137	-	-	-	-	-
Staphylococcus aureus ATCC 64542	+	-	-	+	+
Pseudomonas aeruginosa PTCC 1430	+	-	-	+	-
Klebsiella pneumoniaae PTCC 1290	-	-	-	-	-
(local isolate)Salmonella typhi	+	+	+	-	-
Listeria monocytogenes RTCC 1298	+	-	-	+	+
Escherichia coli RTCC1162	+	-	-	+	+
Shigella dysenteriae PTCC 1188	-	-	-	+	-
Bacillus cereus PTCC 1015	+	-	-	+	-

ATCC: American type culture collection; PTCC: Persian type culture collection; RTCC: Razi type culture collection.

Table 5: Antibiotic sensitivity profile of the tested isolates by disc diffusion assay

Antibiotics	Abbreviations	Conc (mcg)	Zone of inhibition (mm)				
			TA008	TA009	TA010	TA049	RNL26
Chloramphenicol	C	30	23	30	17	21	17
Cefazolin	CZ	30	19	37	23	22	17
Clindamycin	CC	2	21	23	26	22	23
Gentamycin	GM	10	31	20	30	17	22
Vancomycin	V	30	28	17	15	18	11
Ampicillin	AM	10	21	18	17	72	27
Erythromycin	E	15	17	30	23	24	28
Tetracycline	TE	30	20	18	30	22	24
Penicillin	P	10	16	12	22	92	12
Enrofloxacin	NFX	5	33	30	30	22	26
Nalidixic Acid	NA	30	19	23	23	17	9
Amoxicillin	AMX	25	19	13	16	16	24
Oxacillin	OX	1	18	21	17	20	26
Cephalothin	CF	30	18	40	27	11	21
Doxycycline	D	30	25	22	29	22	24
Imipeneme	IPM	10	30	30	30	22	30
Trimethoprim-sulfamethoxazole	SXT	23.75	30	32	30	19	30

Isolates demonstrating zone of inhibition against respective antibiotic discs were recorded as Sensitive (S), Intermediate (I), and Resistant (R) based on their zone diameters. S: $\geq$ 21mm; I: 16- 20 mm, and R: $\leq$ 15 mm. The antibiotic sensitivity profile of the selected isolates is shown in Table 5. Significant differences ($p< 0.05$) were recorded in the antibiotic sensitivity profile of the tested isolates. Among the tested isolates, *B.megaterium* TA008 appeared the most sensitive isolate demonstrating widest zone of clearance against all the tested antibiotics. *L.acidophilus* RNL26 appeared resistant to nalidixic acid and vancomycin, while *B.megaterium* TA009 was resistant to penicillin.

The presence of virulence genes in the bacillus species in study were evaluated by PCR reactions using a set of Bacillus species virulence determinants (Table 6). *B.megaterium T*A009 showed presence of HBL complex genes (*hblA* and *hblC*) giving PCR products of 873 and 399bp, while *B.brevis* TA010 showed presence of only cytK gene (421 bp). The other two bacillus species in study appeared avirulent as none of the tested virulence genes were observed during the PCR reactions.

Table 6: Detection of genotypic virulent determinants in honey isolated bacillus species

Bacterial Species	Virulent genes
B.megaterium TA009	*hblA* (873bp), *hblC* (399bp)
B.brevis TA010	*cytK* (421bp)

IV. Discussion

Honey has gained wide applications in modern medicine where it is known to have antibacterial, antiviral, antifungal, anti-inflammatory, antiproliferative and anticancer properties (Basualdo et al 2007; Johnston et al 2003; Molan 2001; Skiadas and Lascaratos 2001). Moreover, honey possess the dual property of killing bacteria and neutralizing endotoxins and hence know to holds great promise as a new class of antimicrobial and antisepsis agent (Finaly and Hancok, 2004).

Honey has been characterized for its natural bacterial flora which has shown to be one of the factors responsible for antibacterial activity in this viscous liquid. Apart from bacillus species, honey has been considered as a reservoir of probiotic bacteria especially Lactic Acid Bacteria (LAB). In this study we isolated a number of Gram positive bacteria including bacillus and a lactobacillus specie from commercially available local honey samples. Knowing the benefits of bacillus and lactobacillus species in probiotic products we explored the probiotic potential of our honey isolates.

Before a putative probiotic bacterium can begin to fulfil its biological role on the host's health, it must survive against a set of environmental injuries imposed during GIT passage. Tolerance to high acidity (low pH) in the stomach and the high concentration of bile components in the proximal intestine are the major host factors for bacteria to survive in GI tract, making these the main requirements for any culture to be considered probiotics (Havenaar and Hiuslnt Veld, 1992; Schillinger *et al.,* 2005). Our studies showed significant resistance of *B.megaterium* TA008 to relatively low pH for a period of approximately four hours. It is worth mentioning that the typical transit time of food in the stomach is approximately 20 minutes to 3 hours. Bile salt tolerance was described to be related to BSH activity, which is a controversial trait in probiotic selection due to the positive role in lowering of serum cholesterol and the negative effect in increasing the level of undesirable de-conjugated bile salts (Corzo and Gilliland, 1999). Bile acid concentration in intestine ranges from 0.2 to 2% in different persons depending upon dietary intake and health status (Gunn, 2000). Many studies have suggested that survival of probiotic bacteria in acid and bile is highly strain specific (Varnazza *et al.,* 2006). In this study, the isolates appearing acidic pH tolerant were also bile salt resistant. All isolates resisted 1% bile salt during the test period.

The antibacterial activity of honey was first recognized in 1892, by van Ketel (Dustmann, 1978). Esawy and his colleagues (2015), reported the significant role of bacterial community of honey in the strong antibacterial activity. *Bacillus* spp. are effective against gram positive and gram negative bacteria where as their extensive inhibition effect is particularly against gram positive bacteria (Yilmaz*et al.,* 2006). The inhibitory effect could be related to the ability of *Bacillus* to produce antibacterial compounds such as bacteriocin, gramicidin S, polymixin and tyrothricin, which are active against a wide range of gram positive and gram negative bacteria (Ravi *et al.,* 2007). The inhibitory spectrum of the honey isolates in study appeared to be their most significant character, as almost all isolates in study were able to inhibit the growth of most of the pathogens. Various opinion exists about the fact that some probiotic strains might bear resistance to specific antibiotics in order to survive when taken simultaneously with an antibiotic (Charteris *et al.,* 1998). Increasing attention has been recently focused on the links between inappropriate use of antibiotics in farm animal feeding and the emergence of antibiotic resistance microorganism in human (Salyers *et al.,* 2004). In this research, we evaluated the susceptibility profile of the isolates against 18 different antibiotics by disc diffusion assay. The absence of antibiotic resistance can be considered as a positive trait for bacteria used in probiotic food production. Amongst the antibiotics, vancomycin, an inhibitor of cell wall synthesis is the major concern as it is one of the last antibiotics broadly effective against clinical infections caused by multi-drug resistance pathogens due to the presence of D-Ala-D-Lactate in their peptidoglycan (Delgado *et al.,* 2015). As the European Union (EU) Scientific Committee of animal Nutrition (SCAN) guidelines, bacteria used in human and animal feeds should not contain any acquired antibiotic resistance (SCAN, 2005). In our study *Bacillus megaterium* TA008 was found to be sensitive to the most of the antibiotics thereby showing no risk of having antibiotic resistance genes. Resistance of *L.acidophilus* RNL26 to vancomycin and nalidixic acid is considered an inherent trait and the bacteria could be considered safe for use.

Although the present market is filled with probiotic products harboring Lactic Acid Bacteria especially lactobacillus species, in last decade, increasing interest has been arisen for the use of bacillus species as probiotics. One of the most important selection criteria for the bacterial strains intended for use in the food or pharmaceutical industry is the concern for their safety, and a *g*enerally recognized as safe (GRAS) status of isolated organisms has to be confirmed by safety studies prior to being incorporated into feed products (FAO/WHO report,2002, 2007). The significance of bacillus species over majority of LAB as probiotic is their longer viability and survivability in stress conditions. However, a number of reports have shown that isolates of some *Bacillus* species exist that appear to carry toxigenic characteristics and are thus associated with disease and other clinical conditions. In this context, a number of *B.subtilis* group species including *B.subtilis, B.licheniformis, B.amyloliquefacians* and *B.pumilus* have occasionally been associated with food poisoning (Lopez and Alippi, 2010). In this study, one of the *B.megaterium* species (TA009) and *Brevibacillus brevis* TA010 was shown to carry the enterotoxigenic genes and thus might be associated with disease or food poisoning. Similar reports have shown the presence of diarrheagenic genes *bce T* (enterotoxin-T), *hblC, hblD*, and *hblD* in *B.megaterium* isolates from honey (Rowan et al., 2001). The toxic potential of these bacteria might indicate primary source of contamination in the honey samples. Further studies are necessary to completely unravel the pathogenic potential of this species However, *B.megaterium* TA008 and *B.subtilis* TA049 appeared to lack the tested virulence genes and thus could be considered safe for further use as a probiotic.

V. Conclusion

The results from this study indicate that our local honey samples could be a good source of probiotic bacteria. Moreover, the bacillus species *B.megaterium* and *B.subtilis* isolated in this study could be considered a significant probiotic candidate for future application in bio products. Although, the bacillus species in this study showed significantly better performance compared to lactobacillus, yet further detailed investigation are required to approve and assure the advantage of using spore former *Bacillus* instead of lactic acid bacteria

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