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Genotypic and Phenotypic Identification of Probiotic Bacteria with Antibacterial Activity against Resistant Pathogens

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A B S T R A C T

Probiotics are described as live bacteria that, when given in sufficient concentrations, provide health advantages to the host. Probiotic bacteria are the favored microorganism to a diversity of industries. The main purpose of this work was to screen probiotic bacteria for their antibacterial activity against resistance pathogen and Identification them using 16s rDNA. The results showed that, the probiotic bacteria were able to prevent growth of pathogens and exhibited significant inhibition of the growth of the Gram positive and Gram-negative bacteria growth with different diameter of inhibition zone. The eight bacteria strains were identified 2 *Lactiplantibacillus 2 L. plantarum, 3 L.pentosusL. mesenteroide.* This study concluded that these secondary metabolite from probiotic bacteria can be used as a natural antimicrobial agent, added to food formulations to prevent of pathogenic microorganism's growth and can be used as a preventive strategy to delay the onset of pathogenic biofilm growth on catheters and other medical insertional materials, reducing the use of synthetic drugs and chemicals.

Keywords: Probiotic bacteria, Antibacterial, Antimicrobial activity, Resistant pathogens 6s rDNA PCR.

1. Introduction

Probiotic microflora has an essential role in human and animal gastrointestinal systems. In human being, some of probiotic bacteria can be observed as commensal in oral hollow, the gastric tract, and the vagina, and usefully effect these human environments. The normal microflora of numerous fermented foods such as vegetables, milk and meats is dominated by Probiotic bacteria which serve as preservatives by decreasing the pH to4(Stiles & Holzapfel, 1997). Probiotic bacteria are established to produce compounds with antibacterial activity, like bacteriocins, lactic acid and hydrogen peroxide (Velraeds et al., 1996b). These compounds prevent the binding of many urogenital pathogens. Another study by Jehan and Dijlah (2014) proved that surface active derived compounds from *Lactobacillus rhamnosus*had anti-bacterial, anti-biofilm and anti-adhesive properties against some bacteria causing urinary tract infection including *K. pneumonia*, *B.cepacia*,*E. coli* and *S.aureus*. Biofilms are organized infectious groups attached to a surface. Distinct micro- attached organisms in biofilms are rooted in a matrix of typically greasy extracellular polymers, and usually show a phenotype that is differs clearly from that of planktonic cells (Douglas, 2003). Most microorganisms are present in biofilms bound to surfaces and not as planktonic organisms. They are endangered from stress causes and can live in non-optimal conditions (Nikolaev &Plakunov, 2007). One more feature of biofilms is their lesser sensitivity to disinfectant agents such as fluconazole, itraconazole. Ketoconazole, amphotericin B, and lucytosine. Proposed processes of medication resistance are: (i) reduced diffusion of drugs via the biofilm matrix; (ii) restriction of nutrients and reduced growing ratio; (iii) occurrence of continued cells, and (iv) expression of fighting genes (Mukherjee & Chandra, 2004). The aim of this work is to evaluate the effectiveness of Probiotic bacteria present in fermented fruit as antimicrobial agent against resistance pathogen.

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2.Materials and methods

2.1 Isolation of probiotic bacteria and preparation of cell free supernatant

Probiotic bacteria were isolated from different fruit samples, pre-cultured and grown in MRS broth medium containing 5% crude incubated in shaker at 37°C, 120rpm for 72 hrs. CFSs from probiotic bacteria were obtained to evaluate their inhibiting activity against the indicator pathogen strains using the agar well-diffusion test. CFS were sourced from MRS broth cultures by centrifugation (Jouan Br4i, France) at 10,000 g for 10 min at 4°C. To prevent inhibition because of pH reduction due to organic acids, the pH of the CFSs was determined at 6.2 with the use of 1 N NaOH. Any inhibition by hydrogen peroxide was also removed by adding catalase. The CFSs were subjected to filter-sterilization through 0.22 µm pore-size filters (Schleicher &Schüll, Dassel, Germany) (Rodrigues et al., 2006).

2.2 Inhibiting activity of probiotic bacteria cell free supernatant against target pathogen

An agar well diffusion approach as reported by Barefoot and Klaenhammer (1983)was employed to evaluate the inhibiting activity of cell free supernatant from Probiotic bacteria against indicator strains. Probiotic bacteria were grown in MRS broth at 37oC for 24 h and the cells were harvested by centrifuging at 4,000 g for 5 min. The CFSs were utilized for the testing of inhibiting activity. To excluding possible inhibition due to organic acids and hydrogen peroxide (H_2O_2), the CFSs were amended to pH 6.5-7.0 with 5 N NaOH and given treatment with catalase enzyme, respectively. Following the treatment, CFSs were put through filter-sterilization by a 0.22 µm membrane filter prior to the antibacterial assay. To prepare indicator strains, cell cultures of each indicator were grown in broth, strike on agar plates and swabbed on the surface of the correct media for each strain. Wells of 7.0 mm diameter were bored with sterile cork borer and 80 µl of culture CFSs of probiotic bacteria was inserted into each well. Inhibition zones diameters (mm) of individual wells were calculated after being incubated at 37 ºC for 24 h and distilled water was the negative control. Each of the experiments was conducted twice.

2.3 Phenotypic identification of probiotic bacteria isolates by API 50 CHL Kit assay

The API 50 CHL technique was used for the identification of eight isolates. Probiotic bacteria were subculture in 20 ml MRS media then overnight incubation at 30C. The culture was rinsed and resuspended in API50 CHL medium. The McFarland method was used to calculate the suspension's turbidity. API 50 CHL tape wells with bacterial suspension were filled with paraffin oil to generate an anaerobic environment. The tape was incubated at 30oC for 24 hours, and the results were observed after 24 hours and conformation the results 84 hours later. carbohydrates were featured by a yellow color when fermented in medium of carbohydrates. The manufacturer's chart was used to score the color reactions (Conter. et al., 2005). API WEB was used to analyze the results (Bio-Merieux).

2.4Genotypic identification of probiotic bacteria isolates using 16s rDNA

The eight strains of probiotic bacteria were gnomically identified according to the method described by Jarvis & Hoffman (2004). The extraction of total genomic DNA from an overnight culture in 20 ml MRS broth at 30 ºC was done employing the Master Pure™ Gram positive DNA Purification Kit (USA). One ml of overnight culture was subjected to centrifugation 11500 rpm for 10 min at 25oC (Eppendorf centrifuge 5804 R) and the pellet was then retrieved. To the pellet 150 µl of TE buffer was added and subjected to incubation at 37 ºC overnight. 1 µl of proteinase K (50 µg/µl, Sigma) was mixed with 150 µl of gram-positive lysis solution and then added to TE buffered mixture and subjected to thorough mixing. What followed was the incubation of the sample at 65-70 ºC for 15 min and then vertexing at 5 min intervals. The next step was to place the sample on ice for 5 min. Then 175 µl of MPC protein precipitation reagent was added to every sample, followed by vertexing and centrifugation at 13,000 rpm for 10 min at 4 oC (Eppendorf centrifuge 5804 R). The CFSs were then moved to new tubes and the pellets discarded. 1 µl of RNase II (5 µg/µl) was added to each sample followed by thorough mixing. The samples were subjected to incubation at 37 ºC for 30 min; 500 µl of isopropanol was added to the CFS, followed by centrifuging at 4ºC for 10 min at 13,000 rpm (Eppendorf centrifuge 5804 R). Isopropanol was eliminated with an Eppendorf pipette, but the DNA pellet was kept in place. The pellets were washed with 200 µl ethanol 70% and subjected to centrifugation at 5, 000 rpm for 2 min at room temperature. The removal of the ethanol was done with care and the DNA was suspended again with 35 µl of deionized water and stored at -20 ºC for future study.

2.5 Gel electrophoresis

Amplifying the PCR products from universal bacterial primer was subjected to analysis for expected size. Two µl of each amplification mixture was put through electrophoresis in 1.5% (w/v) agarose gels in 0.5 x TEA buffer for 45 min and 110 V. DNA molecular mass marker (250 to 10,000 bp) molecular ladders from 1st Base, Malaysia was the standard. Following electrophoresis staining of the gels in ethidium bromide was carried out and after rinsing the gels were observed and photographs taken with UV transilluminator (Bio-Rad Laboratories, Regrate, Italy). The partial 16S rDNA, Lbp11 and LMM primers sequences were established by 1st Base, Malaysia and comparison was made of the sequences and the databases (Gen-Bank). The sequence was BLAST searched on EZ-Biomedical server to get the exact nomenclature of the isolates. The phylogenetic tree was constructed with MEGA 11 using the neighbor-joining method with a bootstrap value of 1000.

2.6 Statistical Analysis

Results are reported as the mean ± standard deviation of three replicates. Statistically significant differences of the experiments achieved in the various tests were subjected to evaluation by a one-way ANOVA ($P < 0.05$) and Tukey's test. Statistical analyses were carried out with SPSS software (version 22.0) and a significant difference was considered if $P < 0.05$.

3. Results and discussion

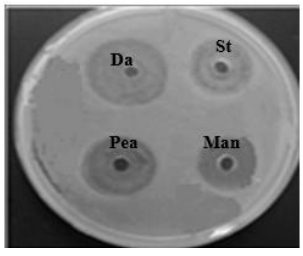
3.1 Antibacterial activity of CFS of probiotic bacteria by Agar well diffusion method

Probiotic bacteria were recorded to have variable degrees of antibacterial activity (Banat et al., 2000, Gudina et al., 2010a). Results of this study revealed that from 20 isolates tested, eight isolates exhibited significant ($p<0.05$) inhibition of the growth of the Gram positive and Gram-negative bacteria growth with different diameter of inhibition zone (Table 1 and Figure 1, 2). The inhibition zone was considered strong (>13mm), moderate (13 to 9 mm) and weak (<9 mm) (Sumathi, 2012). The results showed that four isolates (Da, Pea, St, and Mn had strong inhibition activity against the target bacteria (*Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Proteus vulgaris* with inhibition zone between 17 and 35 mm in diameter. The results of this study concur with those of Rodrigues et al. (2004, 2006). All the target bacteria were strongly inhibited by isolate Da: *Escherichia coli* (35 mm), *Staphylococcus aureus* (32.5 mm), *Pseudomonas aeruginosa* (33 mm), *Klebsiella pneumoniae* (24.5 mm) and *Proteus vulgaris* (26 mm). Secondary metabolite produced by probiotic bacteria have been shown to reduce adhesion of pathogenic micro-organisms to glass (Velraeds et al., 1996a) silicone rubber (Busscher et al., 1996) surgical implants (Ganet al., 2002) and voice prostheses (Rodrigueset al., 2004). Consequently, previous adsorption of these metabolite can be used as a preventive strategy to delay the onset of pathogenic biofilm growth on catheters and other medical insertional materials, reducing the use of synthetic drugs and chemicals (Rodrigueset al., 2007). Moderate to weak inhibition against the target bacteria was observed for isolate Bn, Pe, Apr and Or. Several probiotic bacteria such as lactic acid bacteria strains were reported to possess collagen-binding proteins that induce anti-biofilm activity against different pathogens(Saharan et al., 2014).

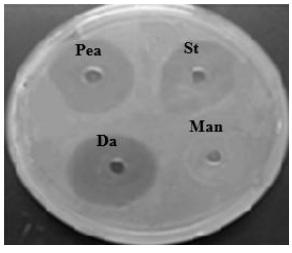
Table 1- Growth inhibition zone (mm) of CFS of probiotic bacteria against target pathogen

Probiotic bacteria	Target bacteria				
	E. coli	***S. aureus***	***P. aeruginosa***	***K. pneumoniae***	***P. vulgaris***
Da	35.0 ± 2.49^{d}	32.5 ± 0.2^{d}	33.0 ± 0.10^{de}	24.5 ± 0.52^{b}	$26.0\pm^{cd}$
Pea	32.7 ± 0.97^{d}	31 ± 0.97^{e}	31 ± 0.63^{d}	22.2 ± 0.4^{b}	26.4 ± 0.11^{e}
St	32.3 ± 0.1^{d}	29.0 ± 0.4^{d}	29.2 ± 0.12^{e}	19.1 ± 0.31^{c}	20.3 ± 1.08^{c}
Man	25 ± 0.01^{f}	27.1 ± 0.1^{f}	28.1 ± 0.20^{f}	18 ± 1.36^{e}	18.1 ± 0.52^{f}
Bn	13.4 ± 0.1^{a}	19.7 ± 0.72^{a}	12.5 ± 1.36^{a}	8.2 ± 0.1^{a}	8.4 ± 0.60^{a}
Pe	11.3 ± 0.84^{c}	13.1 ± 1.08^{b}	10.3 ± 2.22^{b}	12.8 ± 0.71^{b}	9.7 ± 1.07^{d}
Apr	12.8 ± 0.90^{b}	14.4 ± 0.56^{c}	11.0 ± 0.54^{c}	7.1 ± 0.4^{a}	6.1 ± 0.32^{b}
Or	9.8 ± 0.00^{c}	10.4 ± 0.56^{c}	10.0 ± 0.22^{c}	6.1 ± 00^{a}	4.1 ± 0.30^{b}

Different letters in the same row represent significant differences ($p< 0.05$).



Inhibitory zone of probiotic bacteria CFS against *E. coli*



Inhibitory zone ofprobiotic bacteria CFS against *S. aureus*

Fig. 1 - Growth inhibition zone of probiotic bacteria CFS against pathogenic bacteria by well diffusion method

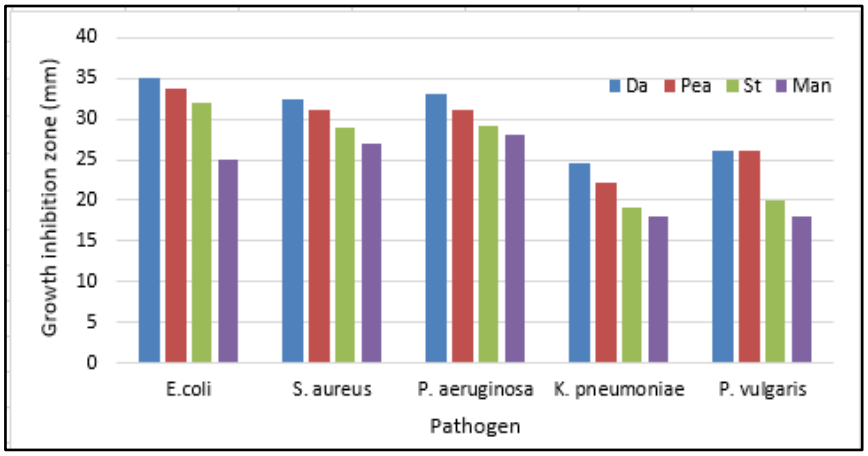


Fig. 2 - Growth inhibition zone of probiotic bacteria against target pathogen

3.2 Phenotypic Identification

According to API 50 CH test kits, eight probiotic bacteria **isolates** (Da, Pea, St, Mn, Bn, Pe, Apr and Or) were identified as *Lactiplantibacillus, L. pentousus, L. plantarum,Lactiplantibacillus, L. pentousus, L. pentousus,L. mesenteroides* and *L. plantarum* with similarity 99.0%, 99.9%, 99.0%, 98.4%, 97.1%, 99.0%, 99.1% and 99.0%, respectively (Table 2).

3.3 Genotypic Identification

Genotype identification of DNA using universal primer indicated clear isolate bands (Figure 3) with estimated molecular weight 1500 bp and similarity (97.0%) for (Da) *Lactiplantibacillus*, (99.0%) for (Pea) *L. pentosus*, (98.6%) for (St) L. plantarum, (97.0%) for (Mn *Lactiplantibacillus*, (98.0%) for (Bn) *L. pentousus*, (99.0%) for (Pe) L. pentousus, (98.0%) for (Apr) *L. mesenteroides* and (99.0%) for (Or) *L. plantarum* (Table 2).

Table 2 -Phenotypic and Genotypic identification of probiotic bacteria Isolates

Code of bacteria	Source	Phenotype Identification		Genotype Identification		
		ID[a]	SI[b] %	ID	SI %	Accession No.
Da	Date	*Lactiplantibacillus*	99.0	*Lactiplantibacillus*	97.0%	KC416993.1
Pea	Pear	*L. pentosus*	99.9%	*L.pentosus*	99.0%	GU451063.1
St	Strawberry	*L. plantarum*	99.0%	*L. plantarum*	98.6%	MH473458.1
Mn	Mango	*Lactiplantibacillus*	98.4%	*Lactiplantibacillus*	97.0%	MT178439.1
Bn	Banana	*L. pentosus*	97.1%	*L. pentosus*	98.0	MZ959488.1
Pe	Peach	*L. pentosus*	99.1%	*L. pentosus*	99.0	MZ959433.1
Apr	Apricot	*L. mesenteroides*	99.0%	*L. mesenteroides*	98.0%	EU419606.1
Or	Orange	*L. plantarum*	99.0%	*L. plantarum*	99.0%	MH473378.1

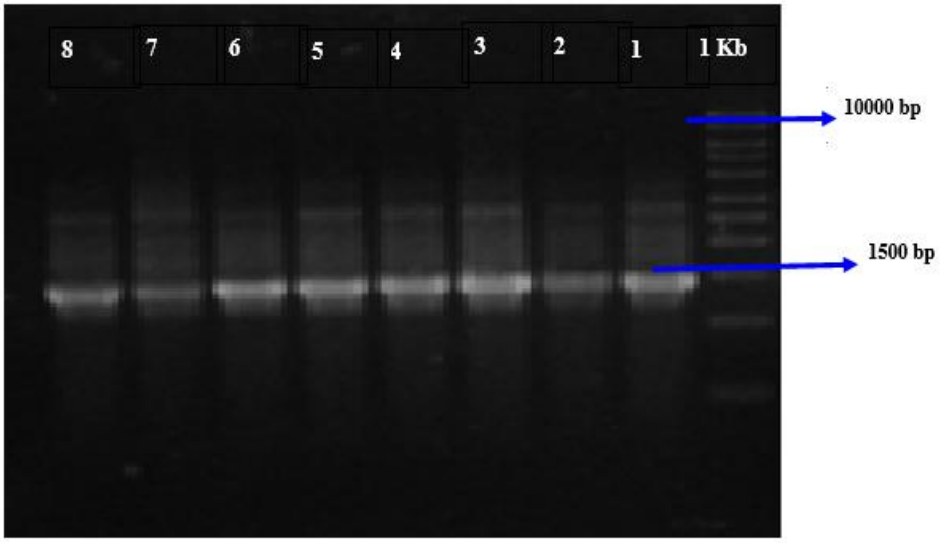


Fig. 3 - The DNA Bands of probiotic bacteriaon the 1.5 % Agarose Gel Using Primers 16S.S:(5-AGAGTTTGATCCTGGCTC-3) and 16S.R. (5-).

The phylogenetic tree was constructed by the Neighbour-joining method (MEGA X 10.0.5). Numbers in parentheses are accession number of published sequences. The numbers at the nodes are bootstrap confidence levels (percentage) from 1000 replicates (Jawan et al 2020). Phylogenetic analysis revealed that probiotic strains have at least 97% similarity with lactobacillus strains (Figure4).

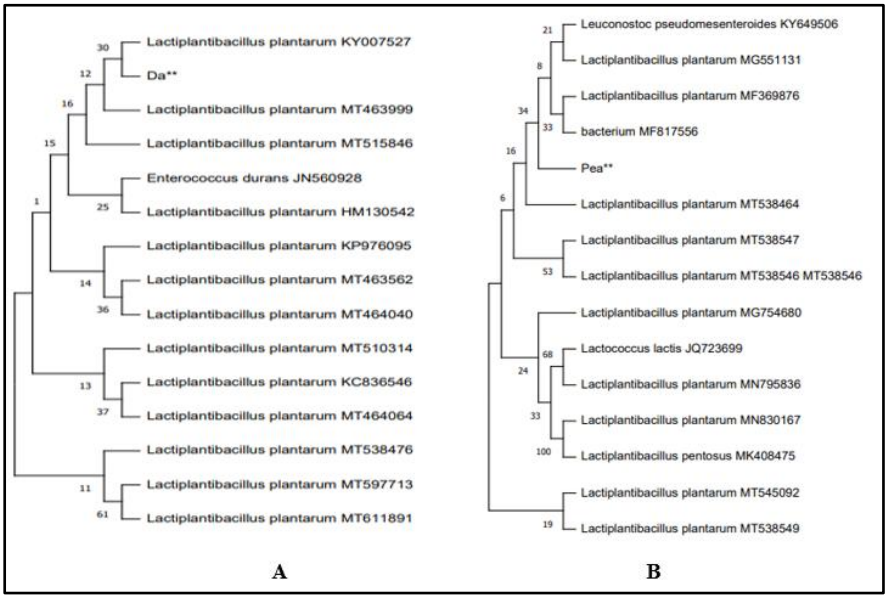


Fig. 4 - Phylogenetic tree of isolates (A. Da and B. Pea) showing the close relatives inferred from 16 S rRNA gene sequence.

The sequences of these isolates were established and kept in the Gene Bank database under accession number KC416993.1, GU451063.1, MH473458.1, , MT178439.1, MZ959488.1, MZ959433.1, EU419606.1 and MH473378.1 respectively (Figure 5).

GGGGATAGCGATACTAGCGATTCCGACTTCATGTAGGCGAGTTGCAGCCTACAATCCGAACTGAGAATGGCTTTAAGAGATTAGCTTACT
CTCGCGAGTTCGCAACTCGTTGTCATCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTATCCCCACCTTCC
TCCGGTTTGTCACCGGCAGTCTCACCAGAGTGCCCAACTTAATGCCAACTGATAATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACAT
CTCACGACACAGCTGACGACAACCATGCACCACCTGTATCCATGTCCCCGAAGGGAACGTCTAACTTTAGATTTGCATAGTATGTCAAGAC
CTGGTAAGGTTCTTCGCGTAGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGCCTTGGGC
CGTACTCCCCAGGCGGAATGCTTAATGCGTTAGCTGCAGCACTGAAGGGCGGAAACCCTCCAACACTTAGCATTCATCGTTTACGGTATGGA
CTACCAGGGTATCTAATCCTGTTTGCTACCCATACTTTCGAGCCTCAGCGTCAGTTACAGACCAGACAGCCGCCTTCGCCACTGGTGTTCTT
CCATATATCTACGCATTTCACCGCTACACATGGAGTTCCACTGTCCTCTTCTGCACTCAAGTTTCCCAGTTTCCGATGCACTTCTTCGGTTG
AGCCGAAGGCTTTCACATCAGACTTAAAAAACCGCCTGCGCTCGCTTTACGCCCAATAAATCCGGACAACGCTTGCCACCTACGTATTACC
GCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAAATACCGTCAATACCTGAACAGTTACTCTCAGATATGTTCTTCTTTAACAACA
GAGTTTTACGAGCCGAAACCCTTCTTCACTCACGCGGCGTTGCTCCATCAGACTTTCGTCCATTGTGGAAGATTCCCTACTGCTGCCTCCCG
TAGGAGTTTGGGCCGTGTCTCAGTCCCAATGTGGCCGATTACCCTCTCAGGTCGGCTACGTA.

Fig. 5 - The complete sequences of bacterial isolates Da isolate from BLAST searched on EZ-Biomedical server

TCATTGCCATGGTGAGCCGTTACCCCACCATCTAGCTAATACGCCCCGGGACCATCCAAAAGTGATAGCCGAAGCCATCTTTCAAGCTCGG
ACCATGCGGTCCAAGTTGTTATCGGGATTAGCATCTGTTTCCAGGTGTTATCCCCCGCTTCCGGGGCAGGTTCCCACGTGTTACTCACCAGT
TCGCCCTCCCTCAATTGGAATCCTGTTGCAAGCCAATTCAATCCAAGGTTCGTTCCATTGCAGGTATAGGCACCGCCACGGTTTCGGGGGG
AACAGAGAACAACCTAAAGCTGCGAATACTAGCGATTCGACTTCTGTAGGCGAGTTGCAGCCTACAATCCGAACTGAGAATGGCTTTAAG
AGATTAGCTTACTCTCGCGAGTTCGCAACTCGTTGTACCATCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGAC
GTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCACCAGAGTGCCCAACTTAATGCTGGCAACTGATAATAAGGGTTGCGCTCGTT
GCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTATCCATGTCCCCGAAGGGAACGTCTAATCTCTT
AGATTTGCATAGTATGTCAAGACCTGGTAAGGTTCTTCGCGTAGCTTCGAATTAAACCACATGCTCCACAGCTTGTGCGGGCCCCCGTCAA
TTCCTTTGAGTTTCAGCCTTGCGGCCGTACTCCCCAGGCGGAATGCTTAATGCGTTAGCTGCAGCACTGAAGGGCGGAAACCCTCCAACAC
TTAGCATTCATCGTTTACGGTATGGACTACCAGGGTATCTAATCCTGTTTGCTACCCATACTTTCGAGCCTCAGCGTCAGTTACAGACCAGA
CAGCCGCCCTTCGCCACTGGGTGTTCTTCCATATATCTACGCATTTCGCACCTACACGTGGGGTTTCCCCGTCCGCTTCTGCGCGCAAGTTT
ACCG.

Fig. 6 - The complete sequences of bacterial Pea isolate from BLAST searched on EZ-Biomedical server

4. Conclusion

A total of twenty probiotic bacteria were isolated from different food sources and screened for antibacterial activity. Out of twenty, eight isolates could produce secondary metabolite with antibacterial activities. Therefore, these secondary metabolite from probiotic bacteria can be used as a natural antimicrobial, antiadhesion and anti-biofilms and can be added to food formulations to prevent of pathogenic microorganism's growth.

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