Full Length Article

INVITRO EFFICACY EVALUATION OF PROBIOTIC PROPERTIES OF BACILLUS ALBUS STRAIN PLAFFS01

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ABSTRACT

Bacillus albus strain PLAFFS01 was screened for in vitro probiotic properties including thermal tolerance, simulated gastrointestinal fluids resistance, hydrophobicity of bacterial surface, autoaggregation, adhesion capacity, antioxidative activity and antibiotic sensitivity. Bacillus albus strain PLAFFS01 was identified by amplifying 1514 bp of 16S rRNA ribosomal gene sequence and the sequence was deposited in the NCBI GenBank database with accession number PP968101. Results showed that the Bacillus albus strain PLAFFS01 was found high survival rate of 97.72% and 79.09% in simulated gastric and intestinal fluids respectively. Also it exhibited sensitivity to antibiotics and good performance on hydrophobicity (64.88%), autoaggregation (41.75%), DPPH free radical scavenging activity (92.68%) and adhesion ability to HT-29 intestinal epithelial cell lines. Moreover, this strain possessed considerable tolerance to thermal treatment, which shows that it can be used in the feed industry also. In conclusion, the present study demonstrated that Bacillus albus strain PLAFFS01 is a potential probiotic candidate.

Keywords: *Bacillus albus* strain PLAFFS01, probiotic characterization

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INTRODUCTION

Probiotics are beneficial microbes that have gained popularity over the years and have found application in the field of human as well as veterinary sciences by several general health and clinical sectors. According to the Food and Agricultural Organization (FAO) of the United Nations (UN) and World Health Organization (WHO), probiotics are live beneficial microorganisms that confer health benefits on the host when it is administered in adequate quantity (FAO/WHO, 2002; Hill *et al.*, 2014). Probiotics play an important role in (a) maintaining a healthy community of microbiota in the host; (b) retrieve the microbiota to a healthy condition; (c)

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producing substances which have beneficial effects; (d) competing with pathogens for their adhesion to the intestinal epithelium and for nutrients; (e) inhibiting the production of bacterial toxins. It can also improve the digestive health (restore the balance of gut bacteria, which can help with gastrointestinal issues), immune response, nutrient absorption, skin health (reduction of inflammation and improve the skin hydration), weight maintenance (preventing the dietary fat absorption in the intestines), etc. Probiotics are not only the effective treatment strategies for treating the various diseases (Al-Khalaifah, 2018; Yang *et al*., 2020), but also safe alternatives of antibiotics to avoid developing antimicrobial resistance (AMR) which is global public health threat by modulating the gut microbiome contests (Eloe-Fadrosh *et al*., 2015; Suez *et al*., 2019).

Searching new bacterial strains of probiotics with numerous inherent attributes are more demand in the treatment of many ailments and disorders (Bazireh *et al*., 2020). The probiotics strain should be confirmed to have certain criteria for the selection of successful probiotics includes thermal tolerance, resistance to gastrointestinal fluid (gastric acid and bile salts), susceptibility to antibiotics, hydrophobicity of the bacterial surface, autoaggregation, ability to adhere to the gastrointestinal mucosa, free radical scavenging activity etc., (Perez *et al*., 1990; Collins *et al.,* 1998; Zarate *et al*., 2000; Ouwehand *et al*., 2002; Xu *et al*. 2023).

Though several promising probiotics strains were reported till date, searching potential probiotic strains is more demand continuously to meet out the standards of scientific, technological and industrial sectors (Marinova *et al*., 2019; Jackson *et al.,* 2019; Binda *et al*., 2020). The present study reported the invitro efficacy evaluation of probiotic properties of *Bacillus albus* strain PLAFFS01.

MATERIALS AND METHODS

Culturing of *Bacillus albus* probiotic

About 1 mg of *Bacillus albus* probiotic spores which is obtained from M/s Sreenivi International KT LLP, Chennai, Tamil Nadu was suspended in 10 ml phosphate buffer saline (PBS) and mixed the content thoroughly for 5 minutes at 250 rpm in an arbitrary shaker.Then 10 µl of this spore suspension was inoculated into nutrient broth and incubated at 37°C for 24 h. From this a loopful of broth culture was streaked on to nutrient agar plate and the plate was incubated at 37°C for 12 h. The culturewas maintained in nutrient agar platesfor further studies.

Identification of *Bacillus albus* probiotic by 16S rRNA PCR

Genomic DNA was extracted by boiling method where by a loop of probiotic colony from nutrient agar plate was resuspended in 100 µl of nuclease free water and was incubated at 100ºC for 10 minutes. Then 900 µl of nuclease free water was added and mixed well until the solution become homogeneous. The DNA was harvested by centrifuging at 12,000 rpm for 10 minutes

and transferred the supernatant to a sterile 1.5 ml microfuge tube. The concentration of DNA was quantified by Nanodrop 1000 ND. For confirming *Bacillus albus*, 16S-rRNA ribosomal gene was amplified by PCR using the forward 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse 1492R (5'-TACGGYTACCTTGTTACGACTT-3') primers (Shinichi *et al*., 2019). The PCR reaction mixture contained 10µl of Taq2XRED Master Mix (AMPLIQON), 10 pmole of forward and reverse primer each and 50 ng of DNA template, brought up to a final volume of 20µL with nuclease free water. The reactions were performed on BioRadT100 thermal cycler under the following conditions: 5 min at 95°C, followed by 30 cycles of 30 sec at 95°C, 1 min at 57°C and 1 min at 72°C; and a final extension step at 72°C for 10 min. Aliquot of 5 µl of PCR product was electrophoresed in 1.5% (w/v) agarose gel. Electrophoresis was performed using 1X TAE buffer, stained with ethidium bromide and run at a constant voltage of 70 V for 2 h. The DNA bands were visualized and images were documented in BioRad Gel Documentation System. The PCR product was purified and sequenced from Eurofins Genomics India Pvt. Ltd., Bangalore. Sequencer was analyzed by searching National Center for Biotechnology Information (NCBI) Nucleotidedatabase using Basic Local Alignment Search Tool (BLAST) web site (http://blast.ncbi.nlm.nih.gov) for similarity between the sequences.

Thermal tolerance

The 12 h grown *Bacillus albus* broth culture was centrifuged at 8000 rpm for 5 min and the cells pellet was resuspended in 1 ml of PBS.Then the cell suspensionwas exposed to various temperatures (50°C, 65°C, 75°C, 85°C, 95°C, 99°C) and the number of colonies in cfu/g was enumerated by pore plate method using 100 µl of heat exposed broth culture.

Resistance to simulated gastrointestinal fluid

The simulated gastric and intestinal fluids were prepared according to the method described by Xu *et al*., (2023) and Zarate *et al.*, (2000) respectively. The simulated gastrointestinal fluids were filter sterilized by 0.22 µm membrane. The resistance assays were performed by inoculating 200 µl of *B. albus* probiotic broth culture into 4 ml of simulated gastric and intestinal fluid. Then the content was incubated at 37°C for 3 h. After that, the number of bacteria was determined by spread plate method on nutrient agar plate. The bacterial viability was calculated by the following formula:

$$\text{Survivability \%} = \frac{(\text{viable count at time t1})}{(\text{viable count at time t0})} \times 100\%$$

Where, t0 means beginning time and t1 represents for 3 h after stimulated gastrointestinal treatment.

Susceptibility to antibiotics

The susceptibility of *Bacillus albus* probiotic against antibiotics was performed by agar well diffusion method (Perez *et al*., 1990). Four hours grown probiotic strain

was spread onto the surface of nutrient agar medium with a swab for the agar well diffusion assay. Three antibiotics with four different levels were used: azithromycin (5, 10, 15 and 25 mg per ml) and amoxicillin (30, 60, 90 and 125 mg per ml) and tetracycline (30, 50, 80 and 120mg per ml). Wells with 6 mm inner diameter in agar plates were prepared and various levels of each antibiotic were added. The plates were incubated at 37°C for 12 h. After incubation, the susceptibility to the antibiotics was expressed in millimeters (mm) of the zone of inhibition (ZOI).

Hydrophobicity of the probiotic bacterial surface

The hydrophobicity of probiotic bacterial surface was determined by the method described by Ahire *et al*., (2021) using an organic solvent xylene. The *Bacillus albus* broth culture was grown in nutrient broth for 12 h at 37°C then centrifuged at 8000 rpm for 5 min. The cell pellet was washed thrice with sterile PBS cell suspension was made with optical density (OD) of 0.85±0.05 at 600 nm. Three ml of probiotic-PBS suspension was mixed by 1 ml xylene and vortexes for 30 s. Then, the content was kept for 30 min at 37°C to separate the organic and aqueous phase. The absorbance of aqueous phase was determined at 600 nm and the hydrophobicity of probiotic bacterial surface was calculated by the formula mentioned below.

$$\text{Hydrophobicity \%} = \frac{(A0 - At)}{(A0)} \times 100\%$$

where, A0 means the value of OD600nm before mixing and At represents for the value of OD600nm after 30min separation in the aqueous phase.

Autoaggregation assay

The autoaggregation ability of *Bacillus albus* probiotic was evaluated by the method described by Del Re *et al*., (2000). The *Bacillus albus* broth culture was grown in nutrient broth for at 25°C for 12 h and the cells were harvested by centrifuging at 8,000 rpm for 5 min. The cell pellet was washed thrice by sterile PBS and resuspended in PBS to adjust the OD 0.85±0.05 at 600 nm. After that, 4 ml of probiotic cell suspension were mixed thoroughly by vortexing for 30 s and kept for 6 h at 25°C without disturbance. Then, the absorbance of probiotic cell suspension was determined at OD600 nm. The auto aggregative ability of *Bacillus albus* was determined by following formula:

$$\text{Autoaggregation \%} = \frac{(1 - At)}{(A0)} \times 100\%$$

where, At represents for the value of OD at 600 nm after 12h incubation at 25°Cand A0 stands for the value of OD600 nm at 0 h.

DPPH free radical scavenging activity

DPPH (1,1-dipheny l-2-picryl hydrazyl) free radical scavenging activity of probiotic, *Bacillus albus* strain was assessed according to the method described by Mu *et al*., (2018) with slight modification. The *Bacillus albus* probiotic was cultured in nutrient broth for 12 h at 37°C and the cells were harvested by centrifuging at 8,000 rpm

for 5 min. The cell pellet was resuspended in PBS buffer after three times washing with sterile PBS and adjusted the cell number approximately 1.0x109cfu/ml. The 2 ml aliquot of probiotic bacterial suspension was added to 0.02 mM of 2 ml DPPH-ethanol solution and incubated in the dark condition for 30 min at 25°C after vortex. The control group includes PBS and 0.02 mM DPPH-ethanol solution and the blank group contained sample and ethanol. The optical density of supernatant was measured at 517 nm after centrifugation at 8000 rpm for 5 min. The DPPH scavenging activity of *Bacillus albus* probiotic was assessed by the following formula,

$$\text{Scavenging activity \%} = \frac{1 - (\text{Abs. of sample} - \text{Abs. of blank})}{\text{Abs. of control}} \times 100\%$$

Hydroxy free radical scavenging activity assay

The hydroxyl free radical scavenging activity of probiotic, *Bacillus albus* was determined by the method described by Xu *et al*., (2023) with slight modification. The reaction mixture (1.0 ml of 20 mM PBS, 1 ml of 2.5 mM 1,10-phenanthroline, 1 ml of 5 mM $FeSO_4$, 1 ml of 3 mM H_2O_2, 1 ml of *Bacillus albus* probiotic cell suspension and 5 ml of sterile water) was incubated at room temperature for 30 min. The absorbance of the mixture was measured at 536 nm after centrifugation at 8000 rpm for 10 min. The hydroxyl free radical scavenging activity of probiotic was determined by following formula.

$$\text{Scavenging activity \%} = \frac{(\text{Abs.of sample} - \text{Abs. of control})}{(\text{Abs. of blank} - \text{Abs. of control})} \times 100\%$$

Adhesion assay

Adhesion ability was measured as per the method described by Jacobsen *et al*., 1999 using HT-29 cell line. The 3 ml of 1x105 probiotic cell suspension prepared with Dulbecco's modified eagle medium (DMEM) was transferred into each well of six-well culture plate and incubated at 37°C in 5% CO_2 till the cell growth attain a minimum of 80% confluence. The spent medium was completely removed 24h before adhesion assay and the cells were fed with DMEM (without antibiotics). The cells were then washed twice with 3 ml of sterile PBS (pH 7.4) and 2 ml of DMEM (without serum and antibiotics) was added and incubated at 37oC in 5% CO_2 for 30 min. Then the probiotic culture (1X109 cfu) suspended in 1 ml DMEM medium (without serum and antibiotics) was added to wells containing a monolayers of HT-29 cells and incubated at 37°C in 5% CO_2 for 2 h. The monolayers were washed five times with sterile PBS (pH 7.4).

Adhesion score: The cells were fixed by adding 3 ml of methanol to each well of six-well plate and incubated for 10 min at RT. The fixed cells were stained with Giemsa stain (0.72% w/v) for 30 min at RT after removal of methanol. Then, the cells were washed with ethanol to remove excess stain. The plate was airdried and examined under oil immersion microscope (Leica, Germany). The number of bacteria was counted in 20 random microscopic fields and were grouped into non adhesive (≤40 bacteria), adhesive (41-100 bacteria) and strongly adhesive (>100 bacteria).

Per cent adhesion: The monolayer cells of HT-29 co-cultured with probiotic culture was detached by using 1 ml of 0.25% trypsin-EDTA solution (Sigma, USA). Then the detached cell suspension was cultured on nutrient agar plates after serial dilution with PBS and colonies were counted (T1 cfu/ml). For control, the probiotic bacteria added initially to six-well plates were also counted (T2 cfu/ml). The percentage of adhesion was calculated using the formula mentioned below.

$$\text{Adhension \%} = \frac{T1}{T2} \times 100\%$$

RESULTS AND DISCUSSION

Identification of *Bacillus albus* strain PLAFFS01 by 16SrRNA PCR:

Total genomic DNA isolated from overnight grown probiotic bacteria (Fig. 1) was used as a template for amplifying 1514 bp of 16S rRNA ribosomal gene by PCR using 16S rRNA universal primers. Speciation for *Bacillus albus* strain PLAFFS01 was confirmed by sequencing 16S rRNA ribosomal gene PCR product (Fig. 2). The phylogenetic tree confirmed the 99% homology of *Bacillus albus* with the query sequence. The nucleotide sequence was deposited in the NCBI GenBank database with accession number PP968101.

Thermal tolerance ability of probiotic bacteria:

The 12 h grown probiotic bacteria, *Bacillus albus* strain PLAFFS01 cells were suspended in PBS was exposed to various temperature ranging from 50°C to 99°C. One of the major problems for probiotic supplementation with feed is thermal deactivation of probiotic bacteria during feed processing and transportation. Therefore, the survival rate of probiotic bacteria after exposing them in heat treatment during feed processing is also an essential property. In our study, *Bacillus albus* strain PLAFFS01 showed high thermal tolerance with viable count 1x107 cfu/ml after 85°C heat treatment, which indicates that this strain can be applied even in feed industry for feed supplementation. But, the number of colonies were decreased with increase in temperature after 85°C.

Resistance of simulated gastrointestinal condition:

The survival ability of *Bacillus albus* probiotic strain PLAFFS01 in gastrointestinal environment such as low pH and bile acid was assessed by inoculating of probiotic bacteria in simulated or artificial gastrointestinal fluids for 3 h at 37°C. After incubation, the bacterial cell suspension was spread on nutrient agar plate and viability was determined. The probiotic strain was exhibited the high survival rate of 97.72% and 79.09% in the simulated gastric and intestinal juices respectively. According to the appropriate pH level of gastric juices for probiotic bacterial screening in avian stomach was ranged from 2 to 2.6. In the present study, the *Bacillus albus* strain PLAFFS01 exhibited excellent abilities to withstand and remain viable in low pH simulated gastric juice as well as high pH simulated intestinal juice, which was in accordance with the previous results of lactobacillus strains (Xu *et al.*, 2023; Jensen *et al.*, 2012; Mota *et al.*, 2006).

Similarly, Golnari *et al.* (2024) reported that 13 strains exhibit above 90% viability among 17 isolates of *Bacillus* sp. evaluated under simulated gastric and intestinal fluids conditions.

Antimicrobial susceptibility:

The antimicrobial susceptibility test was carried out with antibiotics, amoxicillin and azithromycin by incubating the probiotic strain with the respective antibiotic for 12 h at 37°C. The results were shows that the *Bacillus albus* probiotic strain exhibited sensitive to amoxicillin and azithromycin with the ZOI 18 mm and 13 mm respectively (Fig. 3). In this study, there were no concerning intrinsic as well as acquired or transferrable antibiotic resistance was observed with *Bacillus albus* strain PLAFFS01, which exhibits the safety criteria of the probiotic. Golnari *et al*. (2024) evaluated thirty-six spore forming, Gram positive and catalase positive Bacillus isolates for antibiotics sensitivity using twelve antibiotics and the results revealed that there was no antibiotic resistance found in any of the isolates. Whereas, Hong *et al.* (2008) and Green *et al.* (1999) observed only single intrinsic antibiotic resistance to clindamycin, streptomycin and tetracycline on some probiotic *Bacillus sp*. only, not a transferrable resistance which can transfer their resistance to host microbiota, especially pathogenic bacteria, resulting in failure of further treatments.

Autoaggregation and Cell surface hydrophobicity:

The probiotic bacterial strain PLAFFS01 was showed 64.88% hydrophobicity in xylene which indicates the good hydrophobic ability of the probiotic cell surface. According to Mahmoudi *et al*. (2019), the ideal percentage of cell surface hydrophobicity is 40%, as this indicates strong adhesive ability. The auto aggregation abilities of *Bacillus albus* strain PLAFFS01 was determined after 6 h culturing at 25°C with static condition and the result was found to be 41.75 % of autoaggregation ability. There is no single universally defined ‘optimum” percentage of autoaggregation. Anna Zawistowska-Rojek *et al*, 2022 studied autoaggregation among different isolates of Lactobacillus and the values were in the range from 8.4% to 21.4%. Gabriela Krausova *et al.*, 2019 reported autoaggregation capability of several strains of Bifidobacterium and Lactobacillus ranged from 21.7% to 69.7%. Boris *et al.,* 1997 and Del Re *et al.*, 2000 suggested that the autoaggregtion and cell surface hydrophobicity abilities are suitable in vitro assays for the initial nonspecific adhesion of probiotics to the intestinal epithelial layer of the host which pave the way to identify potential adhesive probiotics.

Antioxidative potential:

Antioxidative capacity is an important characteristic of probiotic candidates. In the present study, the antioxidative potential of probiotic strain PLAFFS01 was evaluated by DPPH and hydroxyl free radical scavenging activity assays. The results indicated that *Bacillus albus* probiotic strain PLAFFS01 exhibited 92.68% ability of DPPH free radical scavenging activity and 19.35% of hydroxyl free radical scavenging ability which shows the greater changes on

DPPH radical scavenging activities rather than hydroxyl radical scavenging activities. Previous probiotics reports showed that the host's antioxidant activity is improved by various action, such as stimulating the host antioxidant potential system (Castelli *et al*., 2020), antioxidant metabolites production (Lutgendorff *et al.*, 2008), chelation of metal ions with free radicals (Xu *et al.*, 2023; Lee *et al*., 2005). The present study indicated the presence of high level of antioxidative potential in Bacillus sp. this was in consistence with the previous reports (Xu *et al*., 2023).

Adhesion abilities of *Bacillus albus* strain PLAFFS01 to HT-29 intestinal epithelial cells:

In general, many scientific studies related to the probiotic adhesion abilities were carried out using the Caco-2 and HT-29 cell lines. So, in this study, the adhesion abilities of *Bacillus albus* probiotic strain PLAFFS01 was evaluated by culturing probiotic bacteria on the HT-29 monolayer cell line after 2 h coincubation. The *Bacillus albus* probiotic strain strain PLAFFS01 exhibited as adhesive (41-100 bacteria) in nature under oil immersion microscopic examination (Fig.4) and 42.22% adhesion by enumeration method. Autoaggregation as well as hydrophobicity ability of probiotic bacteria is a phenotypic character related to their adhesion abilities (Kos *et al.*, 2003; Del Re *et al*., 2000). In the present study, the number of probiotic bacteria adhering to HT-29 colon epithelial cell line was investigated by examining them directly under oil immersion microscopic field after staining with Giemsa stain and also enumerating by colony count on agar plate after trypsinization (Charalambos Kotzamanidis *et al*., 2010; Ouwehand *et al.*, 2001). The *Bacillus albus* strain PLAFFS01 exhibited hydrophobicity, autoaggregation and adhesive capacity to HT-29 cell line which indicated their potential ability to colonize the host gut mucosa. Moreover, the probiotic strain also exhibited capacity to bind on polystyrene surface (Fig. 4) which indicates biofilm formation (Jones and Versalovic, 2009).

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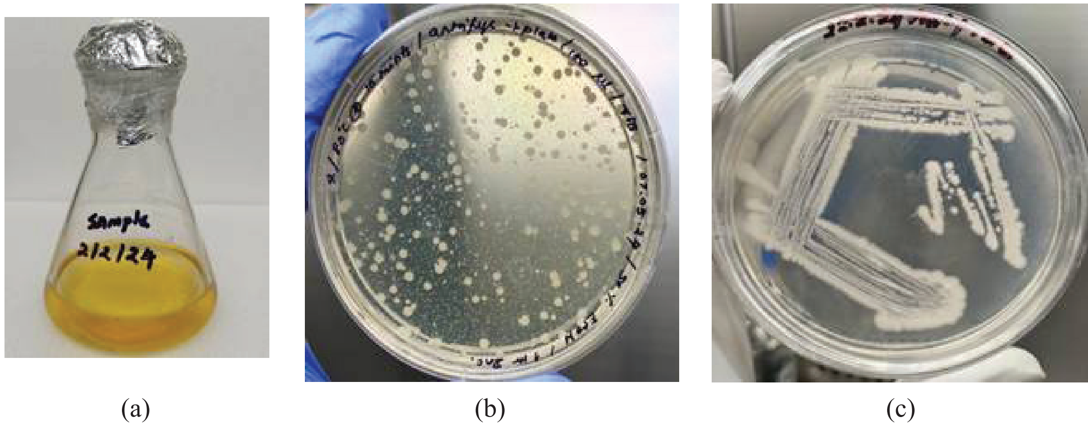


(a) (b) (c)

Fig.1. Culturing of *Bacillus albus* spore probiotic in nutrient medium. (a) Broth culture in nutrient broth medium; (b) probiotic colonies on nutrient agar medium; (c) single colony streaked on nutrient agar medium.

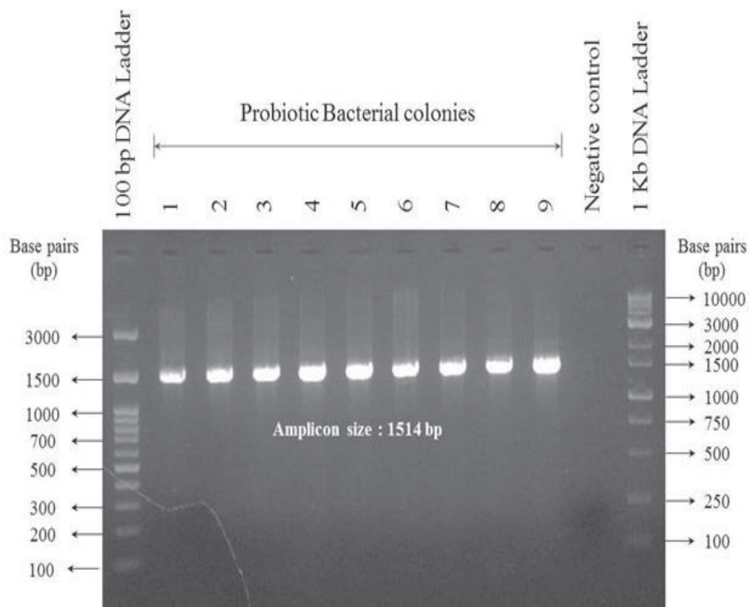
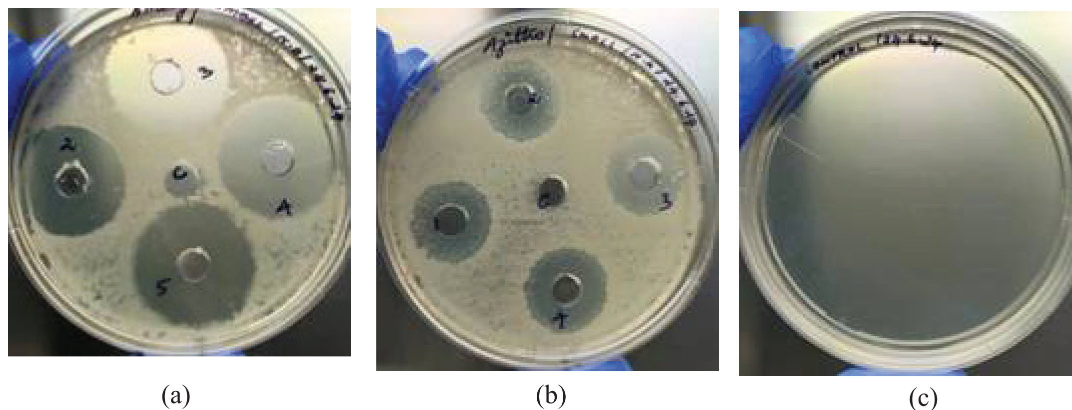
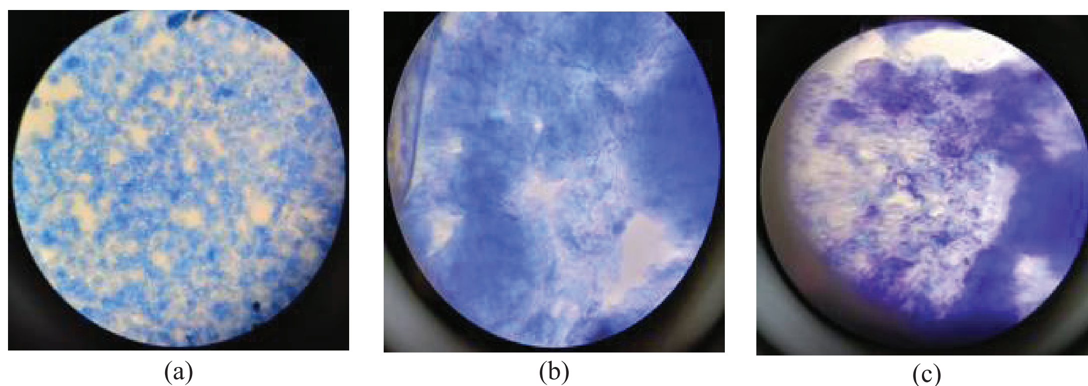


Fig.2. PCR amplification of bacterial universal 16S rRNA gene (1514 bp) from *Bacillus albus* probiotic strain.



(a) (b) (c)

Fig.3. Antibiotic susceptibility test (ABST) on nutrient agar medium. The zone of inhibition of Amoxicillin (a); Azithromycin (b) and Control plate (c).



(a) (b) (c)

Fig.4. Adhesion of *Bacillus albus* probiotic bacteria on HT-29 cell cultures observed under oil immersion microscope (100X) after staining with Geimsa strain. (a) Blank HT-29 cell line, (b) & (c) Bacillus probiotic bacteria on HT-29 cell line culture.

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