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In Vitro Study of Probiotics and their Antimicrobial Susceptibility on Common Pathogens

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Abstract: ***Probiotics are viable bacteria or yeast having beneficial physiologic or therapeutic activities. They showed beneficial effects in several ailments like rota virus, diarrhea, and lactose intolerance, etc. and exhibited anticarcinogenic and immunomodulatory effects. In this study, we have successfully cultured and isolated two strains of probiotics. One of them is Lactobacillus casei, which was isolated from Yakult drink milk from local market of Agra. The other product is the sporlac from which the probiotic strain Lactobacillus sporogenesis was isolated. These two probiotics strains were then subjected to the study on antibiotic susceptibility and detecting the antagonistic activity of probiotic strains. Our results showed that the zone of inhibition was maximum when Lactobacillus casei was cultured under Chloroamphenicol (C30), while the zone of inhibition measured in Lactobacillus sporogenesis was found to be maximum with the antibiotic Levofloxacin (L5). Both strains showed the resistance to the antibiotic Ceftazidime CAZ30. On the other hand, for the antagonistic activity of Lactobacillus casei, the maximum zone of inhibition was observed in the presence of E. coli MTCC-1652 followed by Pseudomonas aeruginosa MTCC-103 and Staphylococcus aureus MTCC-740. As for Lactobacillus sporogenesis, the maximum zone of inhibition was produced against E.coli MTCC-1652 and equal zone of inhibition was observed against Pseudomonas aeruginosa MTCC-103 and Staphylococcus aureus. No zone was seen in the serial suspension for both strains. It is concluded that Lactobacillus sporogenesis produces better zone of inhibition than Lactobacillus casei against Pseudomonas aeruginosa MTCC-103 and Staphylococcus aureus MTCC-740 for some of serial suspension.***

I. INTRODUCTION

Probiotics are living microorganisms that have the abilities to re-balance the intestine bacteria when taken by human or other mammals. Probiotics mainly used from belonging to bacteria although some of them come from yeast. Probiotics are generally regarded as the “good bacteria” as they provide benefits to the health of hosts. A normal family diet may not provide enough beneficial bacteria for human beings. However, maintenance of a proper microbial ecology for human body is required for healthy growth. Probiotics have been proved to play a role in maintaining proper balance by supplementing some kinds of bacteria such as Lactobacillus, Bifidobacterium and Saccharomyces. Now, probiotics are commonly consumed as a part of fermented foods with enough amounts of active microorganisms (Rijkers et al., 2010). Successfully developed food includes yoghurt, soy yoghurt, yakult or some other dietry supplements. It has been suggested that 100 grams of bio-yoghurt or other products with similar biological functions should be consumed every day to benefit from probiotics.

Probiotics are used in multiple different types of digestive problems but since there are several kinds of probiotics but not all will has the benefit that you are looking for it that relates to your health. Possible beneficial effects of probiotics include absorbing and/or destroying toxins from “bad” bacteria, producing substances that benefit human health or boosting the immune system, etc. A good probiotics should be acidic, bile resistant, propagate well in the gut with a strong adhesive capability in the digestive tract of the host and having no side effects. Microorganisms that are commonly used as Probiotics include a) Lactobacillus acidophillus, b) Lactobacillus casei, c) Lactobacillus plantarum, d) Lactobacillus reuteri, e) Lactobacillus gasseri, f) Lactobacillus fermentum, g) Bifidobacterium bifidum, h) Bifidobacterium lactis, i) Enterococcus faecium, j) Bacillus subtilis, k) Bacillus cereus, l) Saccharomyces cerevisiae, m) Saccharomyces boulardii and n) Aspergillus oryzae.

Probiotics interact with epithelial cells and dendritic cells and exert immunomodulatory effect. The animal studies showed that a probiotics organism can modulate the mucosal and systemic immune system(S.Hemiaishwarya.et.al.2013). Stimulation of the immune system in response to rota virus infection was studied with effect of probiotics. Activated monocytes and dendritic cells in the lamina propria produces tumor necrosis factor α (TNF-α) as well as IL-1 and IL-6. Different species of lactobacilli exert different activation patterns on the dendritic cells.

Some probiotic strains produced proteinaceous antibiotic like substances, which were known as Bacteriocin and these are ribosomally coded short peptides and exert Antimicrobial action by interfering cell wall synthesis and by causing pore formation in cell wall of the target organisms.

The antimicrobial attribute of probiotics is related to some active metabolites that is capable of inhibiting and having killing potential for pathogens. As probiotics ferment sugars and produces organic acids such as lactic acid and acetic acid. Thus, the mixture of organic acids has a powerful antimicrobial activity at low pH. In subsequent studies, it was shown that lactobacillus and bifidobacterium, also able to inhibit Helicobacter pylori growth through the release of bacteriocins or organic acid and decrease the ability of this pathogen to adhere to epithelial cells (Prince et al., 2012).

Probiotics also produced secondary metabolites such as ethanol, acetaldehyde, acetoin, CO2 and other germicidal compounds. These metabolites function as growth inhibitory or membrane disrupting factor against pathogens. Similarily "antimicrobial" mechanism of probiotics have been reported in rats exposed to chronic psychological stress (Boirivant and Strober, 2007; Allen et al., 2010). Studies of enteropathogenic E.coli showed that the probiotic organisms Lactobacillus acidophilus and Lactobacillus rhamnosus were able to adhere to a T-84 epithelial cells monolayer and thus inhibit the adhesion of E.coli to this monolayer.(Sherman et al.,2009). Generally, more studies should be carried out on antimicrobial effect by using various kinds of probiotic strains. This study was carried out to examine the antagonistic effect of probiotics strains *lactobacillus casei* and *lactobacillus sporogenesis* on microbial pathogens. We will first isolated these probiotics strains and then studied the antimicrobial effect of these isolated probiotics strains on various common pathogenic micro-organisms.

II. MATERIALS AND METHODS

A. Materials

The probiotic products were collected from the local market of Agra. One is Yakult which is in the form of probiotics drink and another is sporlac which is in the form of powder. The strain Lactobacillus casei is isolated from Yakult and the strain Lactobacillus sporogenesis is isolated from sporlac. Their related information was listed in Table 4.

Lactobacillus casei (L.casei) is a harmless, non-pathogenic microorganism that has been widely characterized and has been found to be beneficial to the human body. It produces lactic acid which helps lower pH levels in the digestive system and impedes the growth of harmful bacteria. L.casei is a type of bacteria that helps to protect the human body from disease and illness by restricting the growth of various types of harmful bacteria that cause infection and be detrimental to individual health. Lactobacillus sporogenesis **(**L. sporogenesis) is a universally occurring beneficial bacteria. It is a gram positive, spore forming, and lactic acid producing probiotic.

Probiotic, such as L.sporogenesis, support the growth of friendly bacteria and help maintain a healthy balance of micro flora in the intestinal environment. L.sporogenesis is better adapted to survive gastric acidity due to its spores. L. spororgenesis supports level of health micro flora thereby improving the gastrointestinal ecology. It also produces only the L (+) form of lactic acid, which is completely metabolized in the body. As the organism grows it assimilates and incorporates cholesterol into its cellular structure, potentially maintaining health lipid metabolism in humans.

Equipment and apparatus used in this study includes Autoclave (Scientific equipment), Hot air oven (Scientific equipment), Electronic analytical balance (Sartorius), Laminar flow (Zenith), Incubator (Toshiba), Deep freeze refrigerator (Sony), Sterile cotton swab tube (Hi-media), Inoculating loop and needle (Hi-media) Micropipette (Torson, Hirsehmann Laborgerate), Trilocular oil immersion microscope (100x) (Nikon), and Glass (Borosil). Glassware used in this study includes Bottle, tubes, petriplates, test tube, conical flask, beaker and measuring cylinder. Before use, they were washed with washing soda as well as chronic acid alcohol and rinsed with clean water and tFhen sterilized in oven at 160˚C to 180˚C for 24hours.

Culture media and their composition were listed below: 1) Muller Hinton Media **(**Beef infusion 300.0 g, Casamino acid hydrolysase of casein 17.5g, Starch 1.5g, Agar 17.0g, Distilled water to 1000ml and pH 7.4); 2) Nutrient agar media (Peptone 5.0g, Beef extract 30g, Sodium chloride 5g, Agar 15g, Distilled water to 1000ml and pH 7); 3) Nutrient broth medium (Peptone 5g, Beef extract 3g, Sodium chloride 5g, Distilled water to 1000ml and pH 7.2); 4) MRS media (de Man Rogosa and Sharpe) (Peptone 1%, Egg extract 0.8%, Yeast extract 0.4%, Glucose 2%, Sodium acetate trihydrate 0.5%, Polysorbate 80 0.1%, Dipotassium hydrogen phosphate 0.2%, Triammonium citrate 0.2%, Magnesium sulphate heptahydrate 0.02%, Manganese sulphate tetrahydrate 0.005% and Agar 1%); 5) Preparation of M.F.S.

To standardize the inoculums density for a susceptibility test, a barium sulphate(BaSO4), turbidity standard, equivalent to 0.5 McFarland standard on its optical equivalent (e.g. latex particle suspension), should be used. A BaSO4, 0.5 McFarland standard may be prepared as 0.5ml aliquot of 0.048mol/L BaCl2 (1.175%w/v BaCl2.2H2O) is added to 99.5ml of 0.18 mol/L H2SO4 (1%v/v) with constant stirring to maintain a suspension. The Barium sulphate suspension should be transferred in 4 to 6ml aliquots into screw cap tubes of the same size as those useful growing or diluting the bacterial inoculums (National Committee for Clinical Lab Studies, 1997).

B. Isolation and Cultivation of probiotic Strains

Probiotic strains were isolated commercially from the probiotic products Yakult and Sporlac. To isolate the *lactobacillus casei* from yakult, a loopful of yakult suspension was inoculated on MRS agar media and kept at 37˚C in an anaerobic chamber. After 48hrs, the pale whitish colonies appeared on the plate which was sub cultured to get the pure culture. To isolate the *lactobacillus sporogenesis* a pinch of sporlac powder was dissolved in 2ml of water and the suspension was inoculated on the MRS agar medium and kept at 37˚C for 24hr. After incubation the colonies were subculture to get the pure colonies. Pure colonies were stored at 4˚C in the butt slants tubes nutrient agar.

C. Maintenance of Test Pathogens

The preservation of the standard strains of the pathogens which are Pseudomonas aeruginosa MTCC-103, Escherichia coli MTCC-1652, Staphylococcus aureus MTCC-740, which were obtained from IMTECH, Chandigarh India. These pathogens were subcultured on the Muller Hinton Media Plates and these plates were further incubated at 37˚C for 24hrs and the stock is maintained for further use.

D. Antibiogram of Probiotic Strains

To detect the antibiotic susceptibility of probiotic strain. The test was examined on the Muller- Hinton media plates against the probiotic strains i.e. one is *lactobacillus casei* and the other is *lactobacillus sporogenesis* for this probiotic suspension of McFarland turbidity 1 was swabbed on the Muller-Hinton Media plate of 90mm diameter. Now the antibiotic disc were placed on the Muller-Hinton plates as which was earlier streaked by the probiotic strains and the plates were incubated at 37C for 24hrs and further the reading were observed.

E. Antagonistic Activity of Probiotic Strains.

Antagonistic activity of probiotic strains was studied with the disc diffusion method according to National Committee for Clinical Lab Studies (NCCLS) guidelines (Kirby et al., 1996). For this petriplates of diameter 0f 90mm is taken on which 20ml of Muller Hinton media is poured in the petriplates, and the plates were allowed to solidify. Now the pathogens, *E.coli* MTCC-1652, *Pseudomonas aeruginosa* MTCC-103 and *Staphylococcus auresus* MTCC-740 were swabbed by using sterile cotton swab stick and the plates were incubated at 37˚C for 15minutes.

Take the sterile disc and absorbed them with the probiotic suspension and soaked them with the probiotic suspension of turbidity equal to M.F.S.≠ 1.0(3 x 10^8 cfu/ml) and the serial suspension of 1/10 (3 x 107cfu/ml) and 1/100 (3x106cfu/ml). Each disc was loaded with 20ml of each probiotic suspension. Now, the disc contain for M.F.S.≠ 1.0 (6 x 106 disc) and the serial suspension M.F.S.≠1/10 (6 x 105 disc) and for serial suspension M.F.S.≠ 1/100 (6 x 104 disc).

Readymade antibiotic disc streptomycin was taken as a positive control and sterile distilled water as negative control. Now allow the disc to absorb their full capacity. After this the plates were taken out from the incubator and place the absorbed disc gently on the surface of the Muller Hinton media. Now put these Muller Hinton agar media plates at 4˚C for 1hr for proper diffusion. After 1hr the plates were kept at 37˚C for 24hours. After 24hrs the zone were measured by using a standard caliper from the back of the test tube. All the tests were done twice in triplet and best was used for readings.

III. RESULTS

A. Isolation and Cultivation of Probiotic Strains.

Probiotic are micro-organisms that some have claimed to provide health benefits when consumed. The probiotic products taken for the bacterium isolation were yakult and sporlac. The strains which were isolated from these two products are *Lactobacillus casei and Lactobacillus sporogenesis.*. They were clearly observed under microscope (Figure 1 A and B).

Lactobacillus casei is a species of genus lactobacillus found in the human intestine and mouth. This particular species of lactobacillus is documented to show the activity under a wide pH and temperature range, and complements the growth of lactobacillus acidophilus, a producer of the enzyme amylase (a carbohydrate- digesting enzyme).

Lactobacillus sporogene is a universally occurring beneficial bacteria. It is a gram- positive, spore forming, lactic acid producing probiotic. Probiotics, such as *lactobacillus sporogenesis*, support the growth of friendly bacteria and help maintain a healthy balance of micro flora in the intestinal environment.

B. Antibiogram of Probiotic Strains

To test the antibiotic susceptibility of probiotic strains (M.F.S. ≠ 1.0), both *lactobacillus casei* and *lactobacillus sporogenesis* were grown under 8 antibiotics as shown in Table 1. These 8 antibiotic discs were placed on the Muller Hinton Media plates which were earlier swabbed by the probiotic strains and the plates were further incubated at 37˚C for 24hr. Then the zone of diameter was measured. In case of *lactobacillus casei,* the maximum zone of diameter was observed with antibiotic Chloroamphenicol (C30), which was measured to be 11 mm and the minimum zone was 4 mm when they were cultured with the antibiotic cefotoxin (CX30) (Table 1 and Figure 2A). For *Lactobacillus sporogenesis*, they showed the maximum susceptibility when they grew under the media with antibiotic Levofloxacin (LF5) and their measured diameter of zone was 11 mm; however, only 3 mm of zone diameter was observed when they were cultured with the antibiotic cefoxitin (CX30) (Table 1 and Figure 2B). Interestingly, no zone inhibition was observed when both *Lactobacillus casei and Lactobacillus sporogenesis* were tested under the antibiotic ceftazidine (CAZ30), which suggested that these two strains were highly resistant to this antibiotic (Table 1 and Figure 2).

C. Antagonistic activity of Probiotic strains Against Pathogens

A total of three different pathogenes including *Pseudomonas aeruginosa* MTCC-103, *Staphylococcus aureus* MTCC-740, *and Escherichia* coli MTCC-1652 were selected to determine the antagonistic activity of probiotic strains against pathogens. These three pathogenes could be well cultured on Agar nutrition media (Figure 3 A-C). The serial suspension of probiotic strains of Mcfarland (M.F.S.≠1.0), (M.F.S.≠1/10) and (M.F.S.≠1/100) were taken. The ready-made disc of antibiotic Streptomycin disc was taken as a positive control and distilled water disc was taken as a negative control. These discs were gently placed on the Muller Hinton Media plates which were earlier swabbed by the pathogens. Then the plates were incubated at 37˚C for 24hrs (Figure 3 D-F for Lactobacillus casei and G-I for Lactobacillus sporogenesis). The results showed that for *Lactobacillus casei*, the maximum zone of inhibition was measured by the serial suspension of M.F.S.≠1.0 against *E.coli* MTCC-1652 followed by the *Pseudomonas aeruginosa* MTCC-103 and *Staphylococcus aureus* MTCC-740(15, 14, 12 mm) and the medium zone of inhibition were observed in the serial suspension of M.F.S.≠1/10 (9mm to 13mm); no zone was seen in the serial suspension of M.F.S.≠1/100 (Table 2 and Figure 4A).

In case *of Lactobacillus sporogenesis, t*he maximum zone of inhibition was produced against E.coli MTCC-1652 (17mm) and equal zone of inhibition against *Pseudomonas aeruginosa* MTCC-103 and *Staphylococcus aureus* MTCC-740 (15mm) in the serial suspension turbidity M.F.S.≠1.0 (Table 3 and Figure 3B). Although the medium zone of inhibition was observed by the serial suspension of M.F.S.≠1/10 (8mm to 15mm). No zone seen in the serial suspension of M.F.S.≠1/100 (Table 3 and Figure 4B). In addition, our experiments also showed that these two strains of bioprotics exhibited the difference in antibiotic susceptibility against 8 different drugs (Figure 5 A and B).

IV. DISCUSSION

This study is carried out to see the antagonistic effects of probiotics strains Lactobacillus casei and lactobacillus sporogenesis against the microbial pathogens *E.coli MTCC-1652, Pseudomonas aeruginosa* MTCC-103, *Staphylococcus aureus* MTCC-740.

For this 3 serial suspension of probiotic suspension (M.F.S.≠1.0), 1, 1/10, 1/100 were prepared, keeping , readymade antibiotic Streptomycin disc as a positive control and distilled water disc as a negative control.

The maximum of zone of inhibition was produced by the serial suspension of Mcfarland turbidity 1.0 followed by the serial suspension 1/10 and 1/100 in all cases of *Lactobacillus casei* produced maximum zone of inhibition against *E.coli* MTCC-1652 (13mm) followed by *Pseudomonas aeruginosa* MTCC-103 (14mm) and *Staphylococcus aureus* MTCC-740 (12mm) by serial suspension M.F.S.≠ 1.0. The medium zones were observed in the serial suspension of M.F.S.≠ 1/10 (9mm to 13mm). No zone was seen in the serial suspension of M.F.S.≠1/100.

Similarly, *Lactobacillus sporogenesis* has also produced maximum zone of inhibition against *E.coli* MTCC-1652 (17mm) in the serial suspension of turbidity M.F.S.≠1.0 and equal zone of inhibition against *Staphylococcus aureus* MTCC-740 (15mm) and Pseudomonas aeruginosa MTCC-103 (15mm*). Lactobacillus sporogenesis* produced inhibition zone of (15, 9 and 8mm) against *E.coli* MTCC-1652, *Pseudomonas aeruginosa* MTCC-103 and *Staphylococcus aureus* MTCC-740 respectively in the serial suspension of M.F.S.≠ 1/10. And no zone was seen against any pathogen by the serial suspension of M.F.S. ≠1/100 turbidity of *Lactobacillus sporogenesis*.

Further the l*actobacillus sporogenesis* produce better zone of inhibition then Lactobacillus casei against the Pseudomonas aeruginosa MTCC-103 (15 and 9mm for M.F.S.≠1 and M.F.S.≠1/10) and Staphylococcus aureus MTCC-740 (15 and 8mm) for M.F.S.≠1.0 and M.F.S.≠1 1/10 respectively. So, for as E.coli MTCC-1652 is concerned, both the probiotics strains produced almost equal zone of inhibition for the serial suspension of M.F.S.≠ 1.0 (17 and 13mm) and for serial suspension of M.F.S.≠ 1/10 (15mm and 11mm) by Lactobacillus sporogenesis and Lactobacillus casei respectively.

Finally it is clear from the above probiotic strain may be used as the complementary and alternative medicine (CAM) for the various infectious disease (Alvarez-olmos, 2001), specially against the resistant pathogens like Staphylococcus aureus and *Pseudomonas aeruginosa etc*.

Probiotics are those live micro-organisms which show the favorable effect on the health. A large species of Lactobacillus, Bifidobacterium, Saccharomyces, Bacillus, Lactic acid bacteria and Escherichia coli are considered as main probotics. This study was carried out to examine the antimicrobial potential of probiotic strains i.e. Lactobacillus casei and Lactobacillus sporogenesis on microbial pathogens-Pseudomonas aeruginosa MTCC-103, Escherichia coli MTCC-1652, Staphylococcuus aureus MTCC-740. Both of the probiotics produces the zone of inhibition against the microbial pathogens. The serial suspension of turbidity M.F.S. ≠ 1.0 shows the maximum zone of inhibition followed by the serial suspension of M.F.S. ≠ 1/10 and no zone in the serial suspension of M.F.S. ≠ 1/100. Moreover the results definitely portrait the probiotics strains as an antimicrobial agent. Though probiotic shows the antimicrobial potential along with it they also show the health benefits when consumed as a regular diet supplements. Nowadays, antibiotics and probiotics combinations are prescribed frequently as these probiotics suppress some health hazards put by antibiotics. At last it is concluded that probiotic as a drug or a food supplements confer a health benefit along with it also protect us from the harmful pathogenic micro-organisms.

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Table 1: Antibiotic susceptibility of probiotic strains used in the study

Antibiotics	Conc./disc (mcg)	Diameter of zone (mm)	
		Lactobacillus casei	*Lactobacillus sporogenesis*
Chloroamphenicol (C)	30	11	10
Cefoxitin (CX)	30	4	3
Azithromycin (AZM)	15	10	8
Amoxycillin (AMC)	20/10	6	7
Ampicillin/sublactam (A/S)	10/10	10	7
Meropenem (MRP)	10	8	10
Ceftazidime (CAZ)	30	0	0
Levofloxacin	5	7	11

Table 2: Antagonistic activity of Lactobacillus casei against pathogens

Antibiotic / Probiotic disc	Zone of diameter(mm)		
	Pseudomonas aeruginosa MTCC-103	E.coli MTCC-1652	Staphylococcus aureus MTCC-740
Probiotic antibiotic	17	20	19
1	14	13	12
1/10	13	11	9
1/100	0	0	0
Distilled water	0	0	0

Table 3: Antagonistic activity of Lactobacillus sporogenesis against pathogens

Antibiotic / Probiotic disc	Zone of diameter(mm)		
	Pseudomonas aeruginosa MTCC-103	E.coli MTCC-1652	Staphylococcus aureus MTCC-740
Probiotic antibiotic	17	24	25
1	15	17	15
1/10	9	15	8
1/100	0	0	0
Distilled water	0	0	0

Table 4: Isolation of Probiotic strains.

Name	Product Information	Composition	Isolated Microorganism
Yakult	6.5 billion lactobacillus strains shirota	Sugar, skimmed milk powder, natural flavours and water	Lactobacillus casei
Sporlac	Spores of lactobacillus sporogenesis	Spores of lactobacillus	Lactobacillus sporogenesis

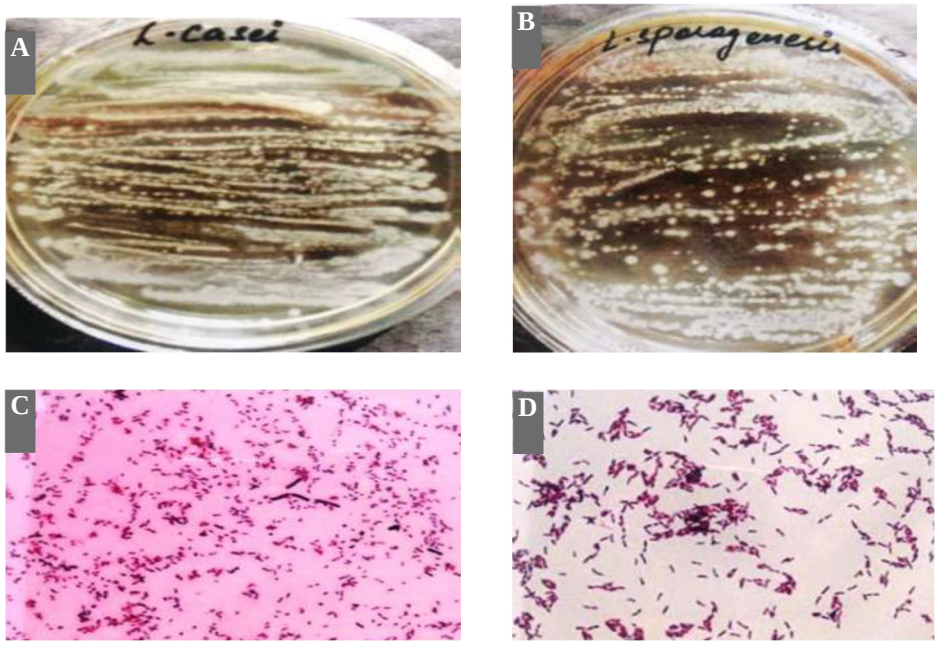
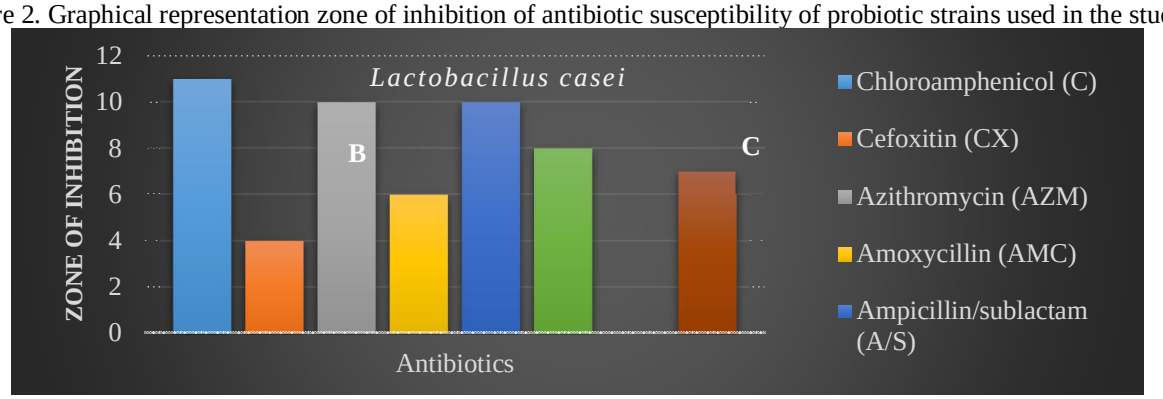
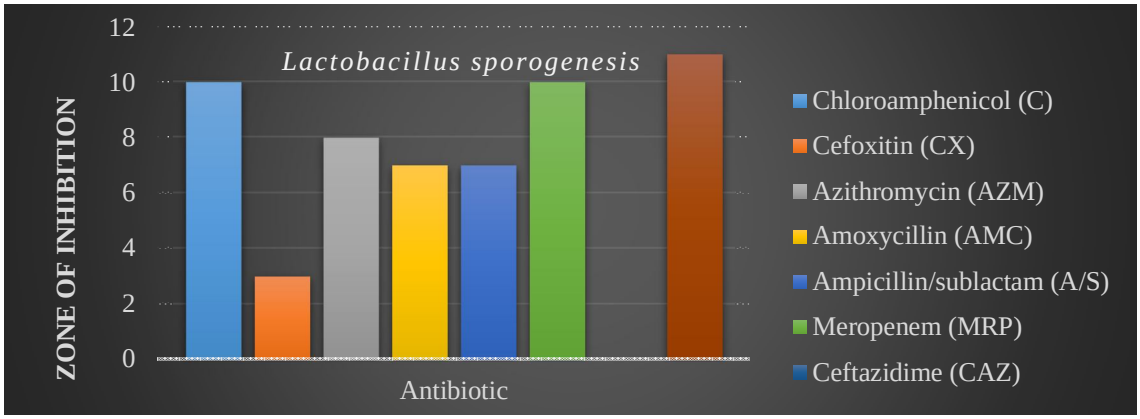


Figure 1. Isolated probiotic strains. (A) and (B) isolated *Lactobacillus casei* and *Lactobacillus sporogenesis*, respectively on agar nutrition medium plates. (C) and (D) showed both *Lactobacillus casei* and *Lactobacillus sporogenesis*, respectively under bright field light microscope with magnification of 100 time

Figure 2. Graphical representation zone of inhibition of antibiotic susceptibility of probiotic strains used in the study. (A)





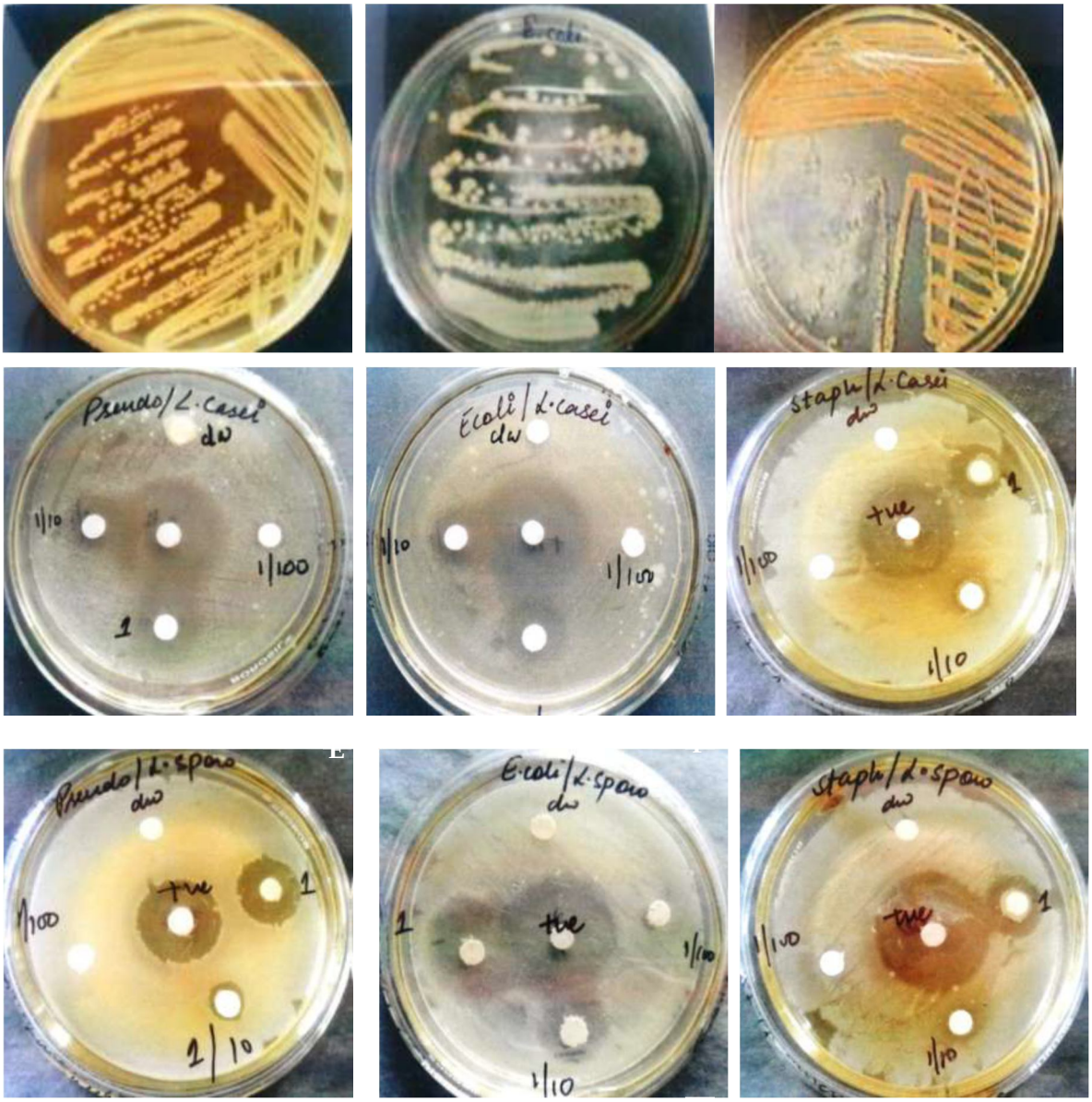
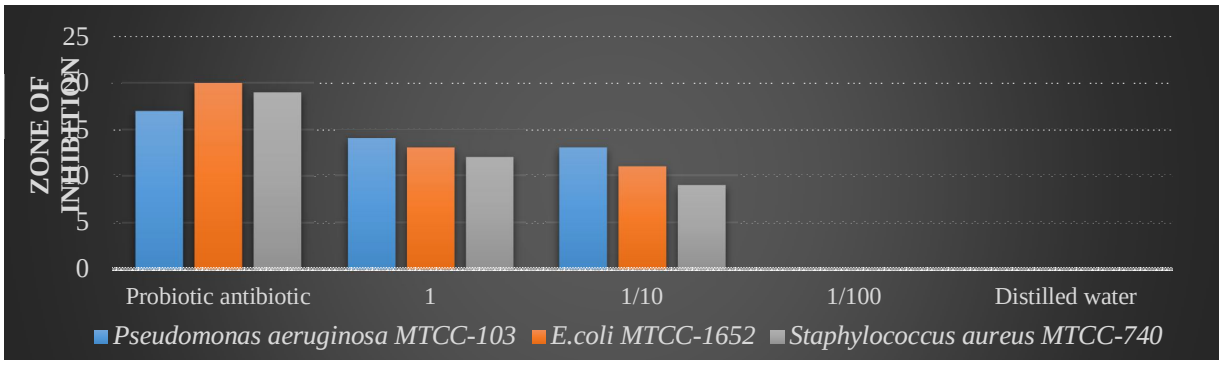


Figure 3. Antimicrobial effect of probiotics against three different pathogenes. (A) to (C) showed the culture of three different pathogenes including Pseudomonas aeruginosa MTCC-103, E.coli MTCC-1652, and Staphylococcus aureus MTCC-740, respectively. (D) to (F) showed the antimicrobial effect of Lactobacillus casei against the Pseudomonas aeruginosa, E. coli and Staphylococcus aureus, respectively. (G) to (I) showed the antimicrobial effect of Lactobacillus sporogenesis against Pseudomonas aeruginosa, E. coli and Staphylococcus aureus, respectively.



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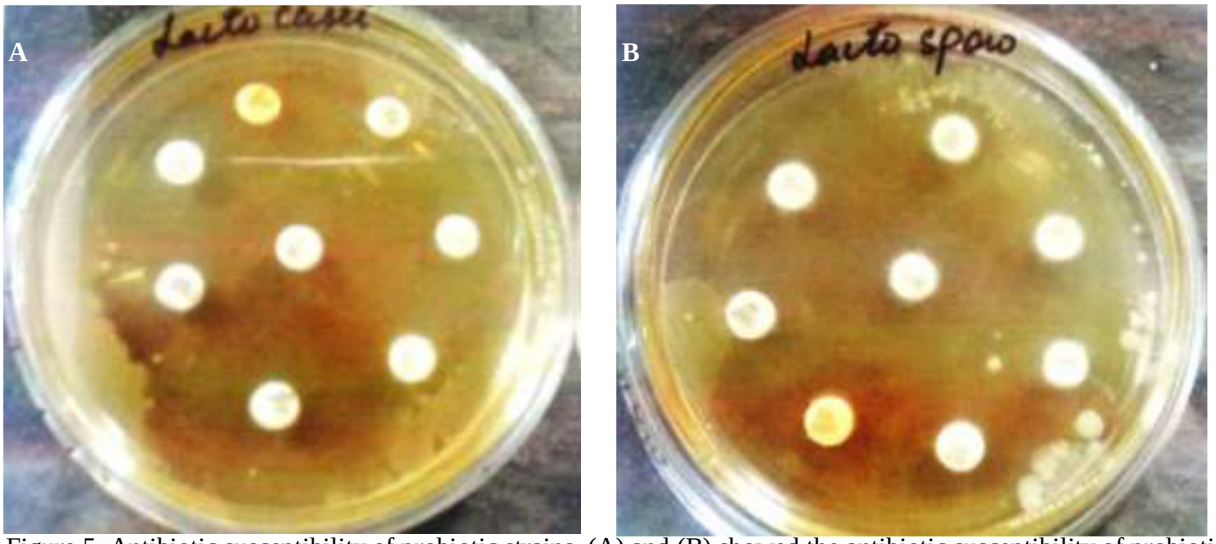
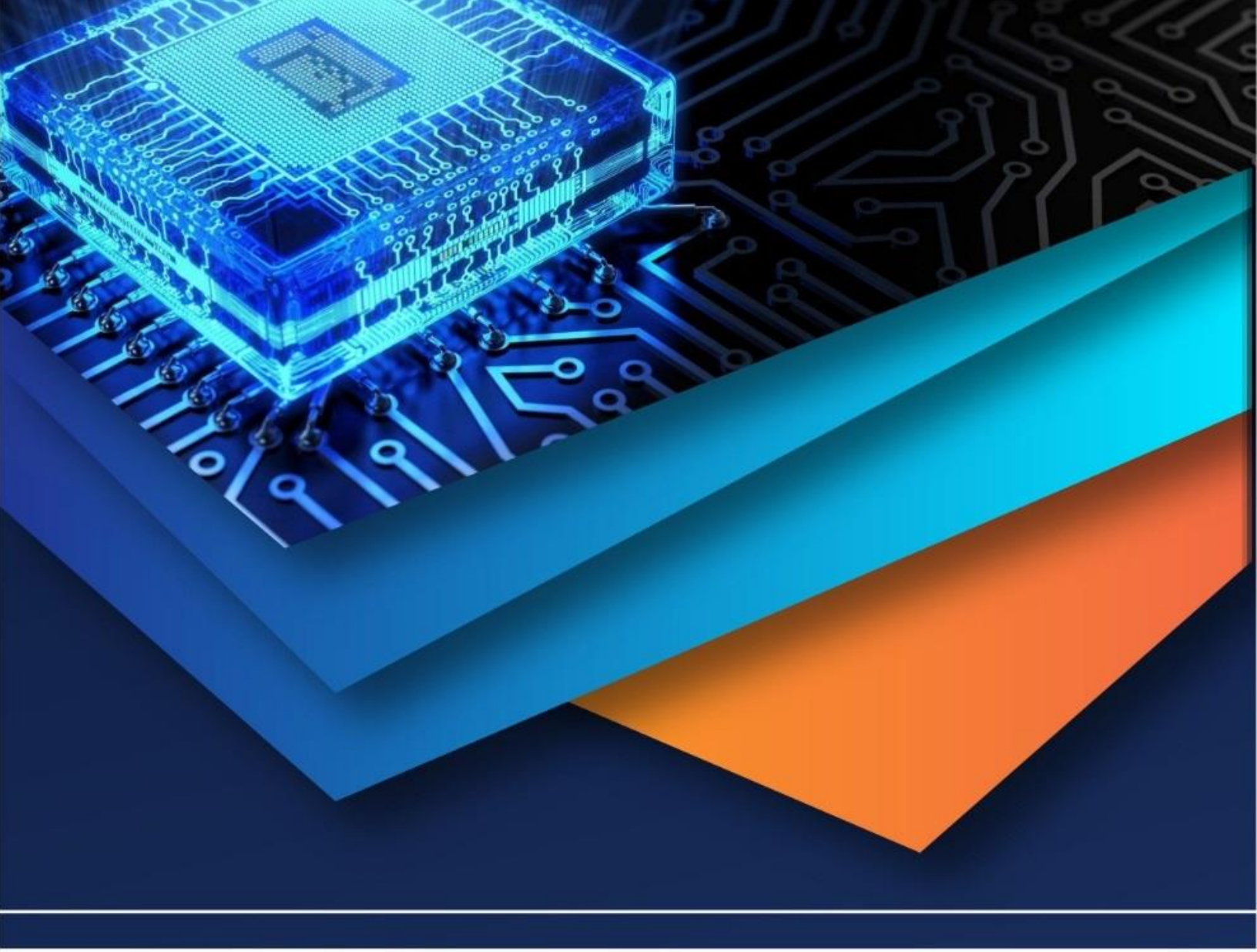


Figure 5. Antibiotic susceptibility of probiotic strains. (A) and (B) showed the antibiotic susceptibility of probiotic strains Lactobacillus casei and Lactobacillus sporogenesis, respectively against the 8 drugs.



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