# CHAPTER 3

# Isolation, Selection and Maintenance of Probiotic bacteria from different source

## 3.1. INTRODUCTION:

Since 20th century the concept of probiotics have been introduced to the society which says that the beneficial bacteria residing in the gut can originate from different sources that includes milk and milk products. In the earliest century the benefits of the curd was widely known & accepted but the use of same as a therapeutic tool was not under consider. Later the researchers reconfirm and re conceptualize the term probiotics as living bacteria confer a health benefit to the host when given in adequate proportions [Hill *et al.*, 2014].

The different kinds of bacteria that remain under the term probiotics include LAB such as, *Streptococcus, Lactobacillus, Bifidobacterium* and *Enterococcus* etc. They are generally present in several milk products, such as curd, yogurt and kefir and also in milk itself [Balakrishnan and Floch, 2012]. The LAB group of bacteria is generally Gram-positive, non-endospore former and catalase negative in nature [Hosseini *et al.*, 2012].

The major role of mechanisms of the probiotics showing their beneficial role to human are; changing the barrier of mucosa, diminishing epithelial cells' apoptotic activity and also increased the secretion of mucin [Gogineni *et al.* 2013; Saad *et al*. 2013]. It initiates elevated synthesis of antibacterial peptides [Mondel *et al*. 2009], and other anti-bacterial substances such as, bacteriocins that modify the gut environment and make it uncomfortable to the intestinal pathogens [Halami et al. 2011; Sharma and Devi 2014], and initiation of a competitive adherence to the epithelial cells [Wu et al. 2000], and thereby helps in intestinal immunomodulation [Tien et al. 2006].

Commercially, probiotics are generally distributed all over the world either as a fermented food or as a diary product. It plays a dominant role as health benefit attributing (Heller 2001). But, the lactose intolerance and high cholesterol are the cases in which the food consumption was shifted towards different items like probiotic fermented vegetables and cereals [Peres et al. 2012; Vijaya Kumar et al. 2015].

Milk is a nutritionally enriched product which is generally produced by almost all domesticated mammals all over the world. Cow milk is an essential commodity for children as well adults in many parts of the world and its products have also gained its importance based on its necessity [Mayo et al., 2010; Tamang et al., 2016a, b, c]. The composition of milk that comprises of several macro and micronutrients includes several proteins, sugars, and fats. Based on the condition of the animals that produce milk, the micro ecosystem has altered to a great extent. The changing lifestyle is also responsible for the alterations of the needs of these products which can again confer some detailed beneficial effects. It can preserve the nutritional value of these products via different mechanisms [Fuller, R., 1992].

In the present study, the aim of our research is to collect different microorganisms from different sources which include milk and milk products (such as, cow milk, human milk, curd and yoghurt). These isolated bacteria were further identified based on some distinct features of the LAB bacteria, i.e.; Gram positive in nature. These selected bacteria were further categorized on the based on their spore producing ability (endospore).

## 3.2. MATERIALS AND METHODS:

### 3.2.1. FINE CHEMICALS:

Microbiological medium used in this study are Nutrient media, MRS media. Chemicals used are crystal violet, Grams iodine, 70% ethanol and saffranine procured from HiMedia, Mumbai, India.

### 3.2.2. INSTRUMENTATION:

The equipments used for the experimental studies were: Bacteriological incubator (Rivotek 50082001, Chennai ), Centrifuge (REMI, Mumbai), Compound Microscope (Olympus CH20i, Greece), Deep freezer (Blue Star, Mumbai), Electronic balance (Shimadzu, Kyoto, Japan), Laminar Air flow (Sanguine Bioinstruments, Hyderabad), Waterbath (Thermoscientific, USA).

### 3.2.3. ISOLATION OF PROBIOTIC ORGANISMS FROM DIFFERENT SOURCES.

For identification the different sources such as; yoghurt, curd and cow milk were obtained from the local market of Purba Bardhaman, 713101. Additionally, breast milk samples of healthy lactating mothers were obtained from local friends and family (23.2324° N, 87.8615° E). Sterile containers are used to collect the samples and transported to the laboratory in ice box. Two samples of each source were taken in account for further experiment.

One gram of curd, yoghurt and one mL of cow milk and human milk was mixed with 9 mL of sterile water and was vortexed for proper mixing. These samples were then aseptically serial diluted upto the dilution rate $10^{-7}$. Then each sample inoculums of all the dilution rates were spread on the nutrient agar and kept for overnight incubation at room temperature in anaerobic condition.

### 3.2.4. SELECTION OF PROBIOTIC BACTERIAL ORGANISMS.

The single colonies of each samples of $10^{-7}$ dilution rate were re-inoculated on MRS agar and were incubated at room temperature in anaerobic condition for 24hours. After incubation the colony morphology and the number of colonies was examined for each of the inoculated MRS plates. Five colonies from each source were selected for further experimental design based on their growth appearance and CFU which was pre calculated for each of the samples.

#### 3.2.4.1. GRAM STAINING:

In 1884, Dr. Hans Christian Gram developed the method of Gram stain which is used to differentiate between two significant groups based on the composition of the peptidoglycan layer and their membrane. The Gram stain reagent is comprised of crystal violet, Grams's iodine solution, ethyl alcohol and safranine; which was applied on the bacterial smear made on glass slide and heat-fixed. The smear of the applied isolates that retained crystal violet and appeared blue were considered as gram positive bacteria whereas, those which retained safranin in place of crystal violet and appeared pink were considered as gram negative.[ Salminen et. al., 1998].

#### 3.2.4.2. ENDOSPORE STAINING:

Ferdinand Cohn has discovered the existence of endospore. Under stress or unfavorable conditions, some bacteria undergo in an inactive stage in which they do not reproduce to remain metabolically inactive. These bacteria possess a structure that is formed inside the cell, hence called endospores. The bacteria possessing these internal spores (Endospore) are generally resistant to external harsh environment which includes heat, chemicals and other agents which are toxic to organisms. In endospore staining procedure the smear of bacteria post heat fixing was placed on water bath at 100°C for 15 mins. flooded with malachite green. After that the

slides containing the smear were immersed in saffranine for 30 secs. Then it was washed under slow running water and observed under light microscope.

### 3.2.5. SAMPLING OF PROBIOTICS AND THEIR STOCK PRESERVATION:

#### 3.2.5.1. PURE CULTURE TECHNIQUES:

The pure culture techniques were performed by using slant technique method on culture tubes containing MRS agar medium. The bacterial isolates were streaked on the slant of the culture tubes for the purpose of sub-culturing and to maintain the purity of the cultures before preserving for further use. [Widdel, 1983]

#### 3.2.5.2. LYOPHILIZATION:

The overnight cultures of the microorganism were grown on nutrient agar plates. The sterile crimp-cap vials with proper labeling were autoclaved prior to experiment. After that, 4 milliliters of lyophilization buffer was added to the plates. If necessary, the cells can be suspended using a autoclaved glass rod. The culture suspension was quickly transported to the sterilized vials (1.5 milliliters per vial) which were sealed with the rubber cap. The culture suspension was freezed inside the vials by placing it in a freezer set at - 20°C. Once the cultures are frozen, it was placed under vacuum condition for stabilization. During the freeze-drying process the vial caps were loosely placed on the top of the vials so that the moisture can escape and the culture was allowed to completely lyophilized (dried out) which takes few hours to overnight depending on the volume of each sample or its volume. The samples were removed from the from the freeze-dryer chamber the vials with the rubber cap and crimp the tops were sealed. And thus the lyophilized culture was stored at room temperature [Strasser et. al., 2009].

### 3.2.5.3. GLYCEROL STOCK PREPARATION:

The glycerol stocks are very important for preserving microbes for a long period. Equal volume (500µL) of overnight bacterial cultures were added to same volume of 50% glycerol stock aseptically and mixed thoroughly keeping the tabs of the vials closed immediately. Paraffin wax tapes are used to seal the vials when stored at -80°C for future uses in different experiments. Post- preservation for the use of glycerol stocks cultures, the vials were opened and re-inoculated on fresh prepared sterile nutrient medium which is then incubated for 48 hours at 37°C to obtain fresh microbial culture [Strasser *et. al*., 2009].

### 3.2.6. ENUMERATION OF SELECTED ISOLATES (PB1 – PB10) AND CALCULATION OF CFU:

The isolates were initially serial diluted with MRS broth upto dilution rate $10^{-5}$. Following spread plate technique, each sample was plated on nutrient agar using 0.1mL inoculums and 24 hours incubations at 37°C. Post incubation, the colonies were counted to calculate CFU (colony forming unit) of each sample.

## 3.3. RESULT AND DISCUSSION:

The gastrointestinal tract possess dynamic micro ecosystem with huge microbial diversity but maintained in an equilibrium state [Collado *et.al*. 2009]. The GI microbiota impart its effect in the maximum availability of the nutrients, generations of new nutrients involved in carbohydrate and protein digestion, withdrawal of toxic compounds acts as an protective layer in the intestinal mucosa, involved in disease in disease protection mechanism trigger up our immune system and facilitates the motor function of the gastrointestinal tract [Guarner and Malagelada 2003; Barbara et.al. 2005].

It is very difficult for the majority of bacteria to colonize stably in the stomach and duodenum due to acidic environment of the stomach, bile and pancreatic juice secretion, oxygen gradients and the motor activity of these areas. So, only a selective number of microorganisms can reside in the GI tract withstanding all the harsh conditions in that area. Thus Collado *et. al.* 2009 recognized intestinal microorganisms as an active organ. Many different microbes resides in the GI tract based on their spatial and temporal aspects [Sekirov et.al. 2010]. Microbial density in the colon also varies in respect of oxygen gradients where the intestinal lumen possesses more bacteria than the epithelial mucosal surface of the intestine. [Espey, 2013].

Considering all the factors stated above we have isolated 67 bacterial samples from four different sources, viz; human milk sample, cow milk, local curd and yoghurt.

### 3.3.1 MORPHOLOGICAL CHARACTERISTICS OF THE ISOLATED SAMPLES FROM DIFFERENT SOURCES ON NUTRIENT AGAR MEDIUM:

The morphological characteristic of all the isolated colonies have been characterized and presented in the **Table 3.1.** Among the 67 isolated colonies which has been isolated from four different sources, it was observed that human milk sample gave seven colonies from one sample and eight from the other sample, again nine from one of the sample of cow milk and ten from the other sample of the same source, and from curd we obtained eleven colonies from one sample and five from the other sample, whereas, yoghurt gave nine and eight single colonies from each of the sample respectively. All the isolated single colonies were observed best at $10^{-7}$dilution factor.

**TABLE.3.1.: Colony morphology of the isolates from different sources ( milk and milk products).**

| SOURCES | DILUTION FACTOR ($10^{-7}$) | | SAMPLES | No. of colonies | Morphological Characteristics of the colony |
|---|---|---|---|---|---|
| | | HUMAN MILK SAMPLE | SAMPLE I | 07 | Round, oval, shiny / off-white and some are pigmented colonies. |
| | | | SAMPLE II | 08 | Round shiny white colonies. |
| | | COW MILK | SAMPLE I | 09 | Round, creamy color, and few flat off-white colonies. |
| | | | SAMPLE II | 10 | Convex, shiny, circular white colonies. |
| | | CURD | SAMPLE I | 11 | Round, white and some flat colonies. |
| | | | SAMPLE II | 05 | Convex, shiny circular colonies. |
| | | YOGHURT | SAMPLE I | 09 | Round, creamy in color and convex white colonies. |
| | | | SAMPLE II | 08 | Round, flat off-white in color colonies. |

The **Table 3.1** showed that most of the single colonies obtained was round in shape, and they are either having shiny appearance or some of them are also having off-white color colonies and very few showed pigmented colonies. Keeping in view of all the morphological characteristics, it was assumed that the colony morphology of the isolates resembles with probiotic bacteria. [Chakraborty and Bhowal, 2015].

### 3.3.2 MORPHOLOGICAL CHARACTERISTICS OF THE SELECTED SAMPLES ON MRS AGAR MEDIUM FOLLOWING THE SAME DILUTION FACTOR (i.e., $10^{-7}$):

The selected isolated colonies were further grown on the MRS selective agar medium to identify the bacteria belongs to the Lactic Acid Bacteria group. Altogether 14 isolates were selected out of which three from human milk sample designated as HMS1 – 3, two from samples of cow milk (CW1 & CW2), five from curd sample which was named as CU1 – 5 and four from yoghurt sample designated as YG1 to YG 4. The morphological characteristic of the selected samples supports the view that they are probiotic in nature. [Hossain and Al-Bari, 2016].

**TABLE.3.2.: Morphology of the colonies on MRS selective medium.**

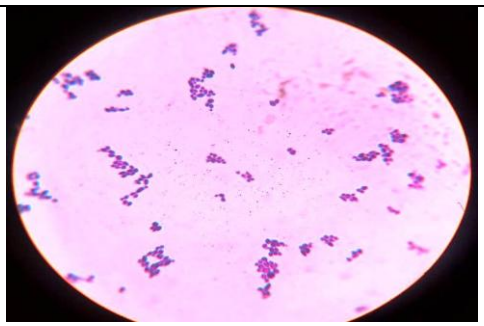
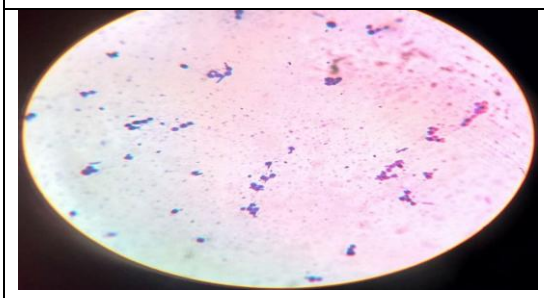
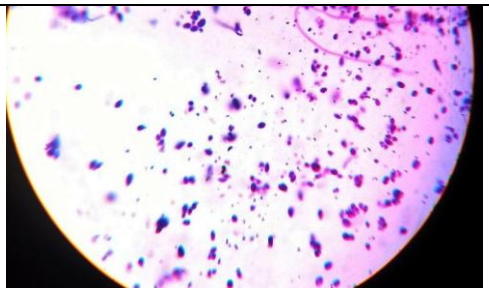
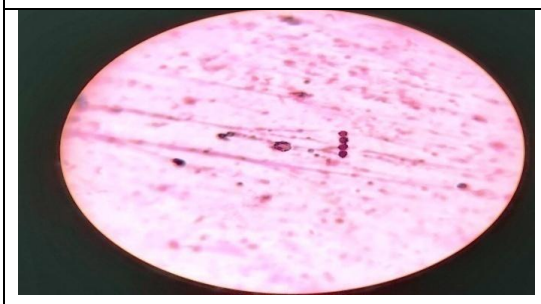
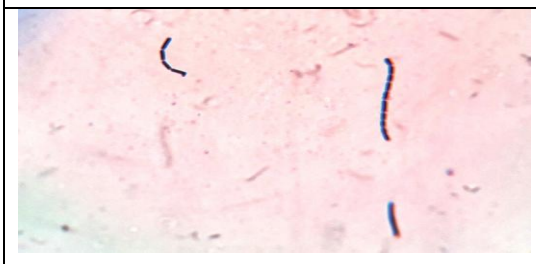
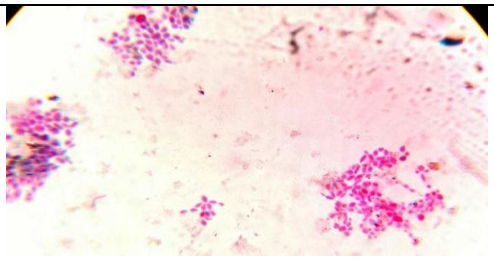
| SOURCE | DILUTION FACTOR ($10^{-7}$) | SOURCES | SAMPLES | No. of samples that grow on MRS agar medium | Morphological Characteristics of the colony |
|---|---|---|---|---|---|
| | | HUMAN MILK SAMPLE | SAMPLE I | 02 (HMS1, HMS2) | Round, oval, shiny colonies. |
| | | | SAMPLE II | 01 (HMS3) | Round shiny white colonies. |
| | | COW MILK | SAMPLE I | 01 (CW1) | Round, creamy color colonies. |
| | | | SAMPLE II | 01 (CW2) | Convex, shiny, white colonies. |
| | | CURD | SAMPLE I | 05 (CU1, CU2, CU3, CU4, CU5) | Round, white colonies. |
| | | | SAMPLE II | 00 | Shiny circular colonies. |
| | | YOGHURT | SAMPLE I | 01 (YG1) | Round, creamy white colonies. |
| | | | SAMPLE II | 03 (YG2, YG3,YG4) | Round, off-white in color colonies. |

### 3.3.3 GRAM CHARACTERISTICS OF ALL THE ISOLATES:

The 14 isolates selected from the MRS agar medium were then introduced to the next set of selection, i.e., Gram Staining method. Out of these 14 isolates, ten isolates have responded in Gram staining as Gram positive strains and were designated as PB1 to PB10. From the human milk sample only HMS3 was observed as a purple rod shaped bacteria under the light microscope and is named as PB7. The samples isolated from the cow milk were two, out of that only CW1 showed Gram positive property and is rod in shape designated as PB6. Besides that, all the samples isolated from curd were selected as Gram positive strains. They are named as PB1 (rod in shape), and the rest of the isolates were PB2, PB3, PB4 and PB5 which were cocci in shape. PB8, PB9, and PB10 isolates (isolated from yoghurt sample) were found to be Gram (+) ve and cocci in shape.

**TABLE.3.3. Gram properties of the selected isolates.**

| SOURCES | SELECTED COLONIES | GRAM CHARACTERISTICS | | DESIGNATED BY NAME |
|---|---|---|---|---|
| **HUMAN MILK SAMPLES** | HMS-1 | (-) ve | Rod shaped | - |
| | HMS-2 | (-) ve | Rod shaped | - |
| | HMS-3 | (+) ve | Rod shaped | PB7 |
| **COW MILK** | CW1 | (+) ve | Rod shaped | PB6 |
| | CW2 | (-) ve | Rod shaped | - |
| **CURD** | CU1 | (+) ve | Rod Shaped | PB1 |
| | CU2 | (+) ve | Cocci Shaped | PB2 |
| | CU3 | (+) ve | Cocci Shaped | PB3 |
| | CU4 | (+) ve | Cocci Shaped | PB4 |
| | CU5 | (+) ve | Cocci Shaped | PB5 |
| **YOGHURT** | YG1 | (+) ve | Cocci Shaped | PB8 |
| | YG2 | (-) ve | Rod shaped | - |
| | YG3 | (+) ve | Cocci Shaped | PB9 |
| | YG4 | (+) ve | Cocci Shaped | PB10 |

| | |
|---|---|
|  |  |
| **PB1** | **PB2** |
|  |  |
| **PB3** | **PB4** |
|  |  |
| **PB5** | **PB6** |
|  |  |
| **PB7** | **PB8** |

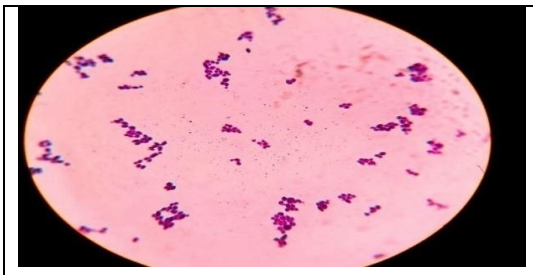
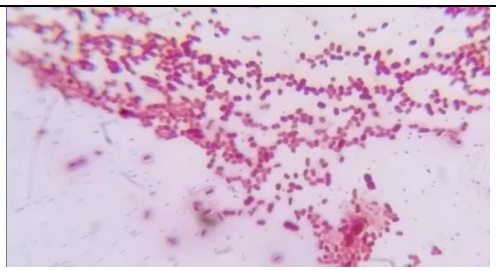
| | |
|---|---|
|  |  |
| **PB9** | **PB10** |

**FIGURE3.1.: Gram staining of the isolates (PB1 – PB 10).**

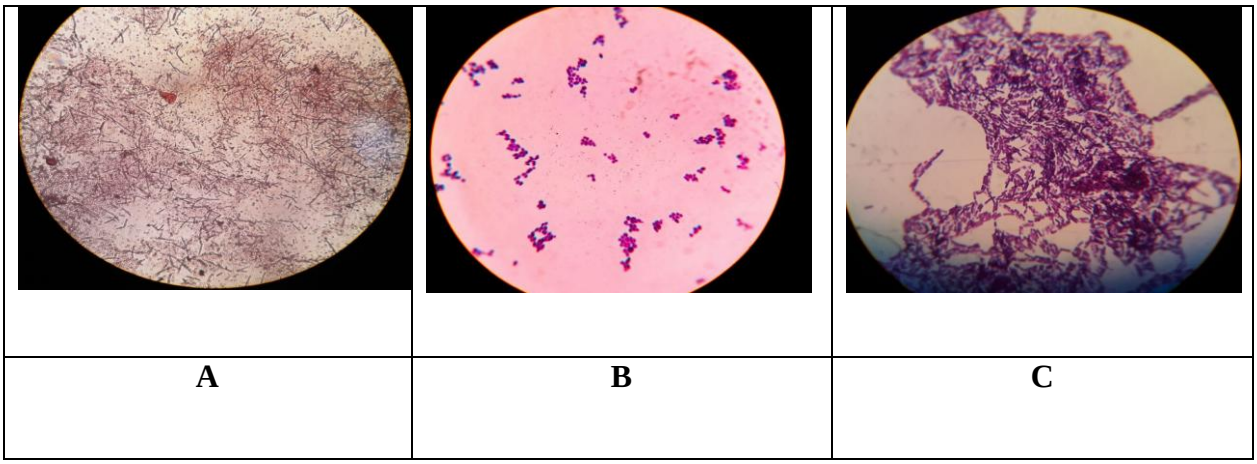
The bacteria such as, *Leuconostoc*, *Streptococcus, Lactococcus* and *Lactobacillus* are present in LAB group which is Gram positive and are facultative anaerobes that can restrict the growth of several harmful pathogens. The isolated strains PB1 to PB10 in this study have been executed as Gram positive cocci, although few were identified as rods in shape. [Sabina Fijan ,2014].

### 3.3.4 IDENTIFICATION OF THE PRESENCE OF ENDOSPORE IN THE GRAM POSITIVE SELECTED ISOLATES (PB1 – PB10):

The group of bacteria that is aimed to isolate in this study is generally included in the LAB (Lactic Acid Bacteria) group which is a non-spore former. But there are different groups of bacteria which despite of being a spore former have the potentiality to be considered as a probiotic. [Elshaghabee and Fouad, 2017]. Based on the above facts the endospore staining is not included in the selection procedure of probiotic bacteria in the present study, rather it is considered as confirmatory characteristics of the selected Gram positive isolates which can be a putative probiotic.

**TABLE.3.4: Endospore forming characteristics of the selected isolates.**

| ISOLATES | ENDOSPORE FORMING CHARACTERISTICS |
|---|---|
| PB1 | Endospore Former |
| PB2 | Non- Endospore Former |
| PB3 | Non- Endospore Former |
| PB4 | Non- Endospore Former |
| PB5 | Non- Endospore Former |
| PB6 | Endospore Former |
| PB7 | Non- Endospore Former |
| PB8 | Non- Endospore Former |
| PB9 | Non- Endospore Former |
| PB10 | Non- Endospore Former |



| A | B | C |
|---|---|---|

**FIGURE.3.2.: Endospore forming characteristics: A; non-spore forming rods, B; non-spore forming cocci bacteria, C; Spore forming rods.**

### 3.3.5. ENUMERATION OF ALL THE SELECTED ISOLATES (PB1 – PB10) AND CALCULATING THEIR CFU COUNT:

The CFU count is an essential factor for the microbes to enumerate the total count of viable cells of the selected isolates which can be used further for therapeutic uses in different combinations. CFU known as colony-forming unit is a parameter to express the number of living cells in a given sample [Minnen and Paul, 2007].

**TABLE.3.5.: The Colony Forming Unit of the isolates (PB1 – PB10).**

| PROBIOTICS | No. OF COLONY | TOTAL CFU = Total colony/ (vol. of inoculums X dilution rate) |
|---|---|---|
| PB1 | 135X4 = 540 | $540 / 0.000001 = 0.5 \times 10^9$ |
| PB2 | 100X4 = 400 | $400 / 0.000001 = 0.4 \times 10^9$ |
| PB3 | 117x4 = 468 | $468 / 0.000001 = 0.4 \times 10^9$ |
| PB4 | 229X4 = 916 | $916 / 0.000001 = 0.9 \times 10^9$ |
| PB5 | 178X4 = 716 | $716 / 0.000001 = 0.7 \times 10^9$ |
| PB6 | 191X4 = 764 | $764 / 0.000001 = 0.7 \times 10^9$ |
| PB7 | 161X4 =644 | $644 / 0.000001 = 0.6 \times 10^9$ |
| PB8 | 70x4 = 280 | $280 / 0.000001 = 0.3 \times 10^9$ |
| PB9 | 80x4 = 320 | $320 / 0.000001 = 0.3 \times 10^9$ |
| PB10 | 91X4 = 364 | $364 / 0.000001 = 0.4 \times 10^9$ |

**Table 3.5.** showed the number of colonies present in the MRS agar plates inoculated with 0.1 mL of the selected isolates (PB1 - PB10) at $10^{-5}$ dilution rate. Maximum count was observed in PB4 and others also exhibited a moderate amount of colonies, thereby the isolates have the potentiality for better growth and can be preserved for a extended period of time for further therapeutic studies [ Kligler *et.al.*, 2008].

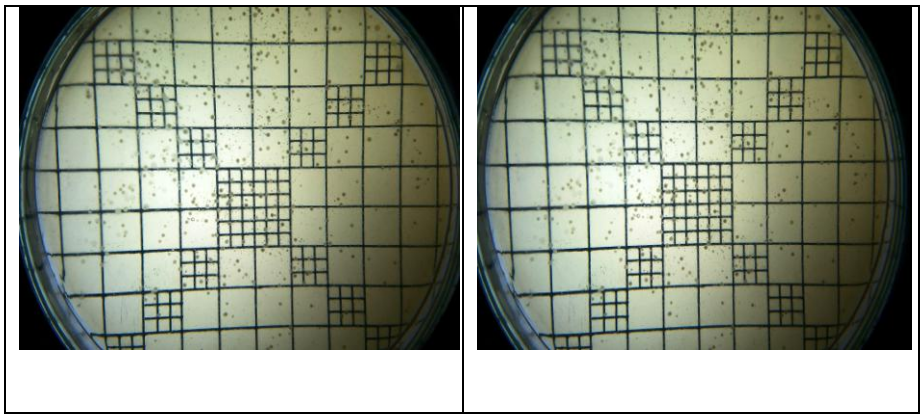


**FIGURE.3.3. The observation showed the single colonies that was obtained for the different isolates on MRS agar media which was placed on the Colony counter device.**

### 3.4. CONCLUSION:

Probiotics are the good microbes that may contain a variety of microorganisms most commonly Lactic Acid Bacteria (LAB) group of bacteria. The experimental results obtained from the present study exhibited that Lactic acid Bacteria was predominant in micro-flora of the samples (human milk, cow milk, curd and yoghurt) studied. The highest LAB colony count was observed in PB4 isolates (isolated from curd sample) although significant numbers of colonies were also observed in the other isolates. Therefore, it can be concluded that all the isolates (PB1 –PB10) have the good characteristics to be considered as a probiotic bacteria.