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FORMULATION AND EVALUATION OF PROBIOTIC CHOCOLATE

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Keywords:

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ABSTRACT: Human gut serves as a home to over 100-1000 microbial species, which primarily modulate the host internal environment and thereby, play a major role in host health. Humans are a unique reservoir of heterogeneous and vivacious group of microbes, which together forms the human-microbiome superorganism. Probiotic bacteria are beneficial to human health by improving the gutmicro biota balance and the defenses against pathogens. Additional health benefits attributed to probiotics are the stimulation of the immune system, blood cholesterol reduction, vitamin synthesis *etc*. Chocolate is mostly a unique food product for all age groups. The addition of lactobacillus to these chocolates thus creates a tastier option for transferring these bacteria to the stomach for the betterment of the microbial environment of the gut. Thus, Lactobacillus and Bifido bacterium were collected from five different samples and introduced into the milk chocolate for the preparation of probiotic milk chocolate. Both the organism was isolated and identified. Their physiochemical properties, sensory attributes and probiotic viability in milk chocolates were assessed. Thus, the study concludes that probiotic milk chocolate can be potentially used as a novel carrier of Lactobacillus and Bifid bacterium with optimum viability and unaltered physiochemical properties.

INTRODUCTION: The United Nations Food and Agriculture Organization estimated that nearly 870 million people of the 7.1 billion people around the world are suffering from chronic undernourishment. Supplementation with probiotics, prebiotics, and symbiotics have promising results against various enteric pathogens. Functional food fits the picture since functional foods are the kind of food that provides benefits beyond basic nutrition.

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Probiotics are beneficial microorganisms improving the intestinal microbial balance in the host when administrated into the gut in sufficient numbers. Probiotic food products have been used worldwide and they are gaining increasing popularity day by day.

Current trends in the consumption of probiotics are associated with increased levels of health-consciousness and the availability of probiotics in the form of dietary supplements. Probiotics are available in various forms including food and beverages, powders, effervescent and capsules. Dairy and non-dairy food products including soy products, cereal-based products, fruit and vegetable juices, fermented meat and fish products are some of the popular probiotic carriers. Chocolate is becoming increasingly popular as a carrier

delivering probiotics to gut [1]. In recent years, the global market of functional foods, especially supplements with probiotics, is expanding rapidly. Probiotics are the live microorganisms that, when administered in adequate amounts, provide health benefits to consumers. The health benefits of probiotics include preventing infectious diarrhoea, decreasing cholesterol levels in the blood, reducing symptoms of lactose intolerance increasing immunity to specific diseases, and acting as an antitumor/anticancer agent [2].

Probiotic organisms which are used in the food must have the ability to stay alive in gastric juices and they should be resistant to the exposure to the bile in the human gut. In addition, they must have the ability to flourish and colonize the digestive tract of humans [3]. In general, the food industry had applied the recommended level of 10^6 CFU/gm at the time of consumption of lactobacillus acidophilus, bifido bacteria and other probiotic bacteria [4]. Yoghurt/curd is the most popular fermented dairy product in India, prepared using mixed mesophilic cultures that ferment lactose to lactic acid. New technology facilitating the supplement of probiotic [lactic acid bacteria, bifidobacterium] in confectionery will be a health promoting ingredient and will be capable in reducing civilization disorder [5].

As the demand for probiotic food products from both dairy and nondairy sources continues to grow, chocolates, one of the most appealing products among the consumers, are anticipated to be an excellent probiotics carrier. Chocolate possesses a wide range of potent antioxidants and other nutrients that can positively benefit human health. In addition, chocolate may serve as a suitable carrier for probiotic delivery to the human gut [6]. Chocolate is one of the most popular confectionary foods due to its pleasant flavor and positive effect on emotions. The milk chocolate system comprises solid particles (cocoa, sugar, and milk powder) dispersed in the fat phase (cocoa butter) [7]. Notably, milk chocolate is known to be a source of a variety of physiologically active compounds with significant antioxidant activity, such as flavonoids and polyphenols. Furthermore, cocoa butter, the lipid portions of chocolate, preserves the probiotic bacteria that promote health benefits.

Targeting gut microbiota is essential for the development of strategies for health care. In this context, Probiotics have potential in the treatment of neurological problems. Likewise, it has been found that the addition of lactic acid probiotic bacteria in functional products helped improve cognitive function in Alzheimer's patients. In addition, the decrease of probiotic strains, such as *L. plantarum* have been associated with cognitive impairment [8].

The most used probiotics include Lactobacillus and Bifidobacterium. Lactic acid bacteria are characterized as gram positive, usually non-motile, non-sporulating that produce lactic acid as a major product of fermentative metabolism. Bifidobacterium is an anaerobic, Gram positive, non-spore forming, pleomorphicrod and was originally named *Bacillus bifidus* commun is producing lactic acid and acetic acid (bactericidal action) as the main products of glucose utilization. [9] Lactobacillus brevis and Bifidobacterium have been shown to be more stable in milk chocolate than probiotic powders or liquids or beverages. The supplementing of the novel Lactobacillus acidophilus probiotic cells into milk chocolate without encapsulation would be a new knowledge [10].

MATERIALS AND METHODS:

Selection of Organisms: Two strains of probiotic microorganisms were chosen they are Lactobacillus sps and Bifidobacterium sps, which are abundantly present in dairy products and fermented foods.

Collection of Samples: The samples were collected from A+curd, Milky mist curd, Goat milk and Zebiotic capsules. A+curd, Milky mist curd, Goat milk as in **Table 1** were procured from supermarket. Whereas zebiotic capsules were obtained from Apollo pharmacy, Madhapur. The samples are shown in **Fig. 1**.

TABLE 1: LIST OF THE SAMPLES USED FOR THE STUDY

Samples	Microorganisms
A+curd	Lactobacillus acidophilus
Milky mist	Bifidobacterium and Lactobacillus acidophilus
Goat milk	Lactobacillus acidophillus
Zebiotic capsules	*Bifidobacterium bifidum*

FIG. 1: PICTURES OF SAMPLES USED FOR THE STUDY

Nutrient Agar Media: Nutrient agar media is used as a medium for the growth of a wide variety of non-fastidious microorganisms. It consists of Beef extract, Peptone, Sodium chloride and Agar as shown in **Table 2.**

TABLE 2: COMPOSITION OF NUTRIENT AGAR

Ingredients	Amount (Gm\L)
Beef extract	3.0
Peptone	5.0
Sodium chloride	8.0
Agar	15.0

Preparation of Nutrient Agar Media: 2.8gms of the nutrient agar was dissolved into 100ml of distilled water and heated until the crystal clear appearance was observed and was sterilized by autoclaving at 15 lbs pressure (121°C) for 15 min. The media was subjected to cooling at 45-50°C and was poured in to sterilized petriplates.

Identification of Organism:

Isolation Sample: Each sample was taken in a sterile container separately and placed in a polyethylene bag during transportation to the laboratory employing standard conditions for sample collection. One gram of curd sample was immediately processed under aseptic conditions by suspending in 9mL of normal saline (0.85%) and was vortexed for proper mixing. Before inoculation, the pH of was adjusted to 6.5±0.2. Each sample was serially diluted, in sterile distilled water. The dilution used for the study was 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}.

Sub-culturing: The colonies obtained were sub-cultured on other plates to obtain pure cultures.

Inoculation of the Organisms: The medium selected for the growth of organisms was nutrient agar medium. A loop full of all the curd samples were streaked on the by quadrant streaking method, under aseptic conditions.

Incubation: All the petriplates were incubated at 37°C for 24 to 48 hrs. All the samples were subjected to evaluation for the confirmation of the organisms.

MRS Broth: The most suitable media for the growth of all Lactobacillus sps. and Bifidobacterium sps. is MRS media. This media supports the luxuriant growth of all lactobacillus from oral cavity, dairy products, foods, feaces (American Public Health Association, 1978) and other sources. The composition of MRS broth is given in **Table 3.**

Proteose peptone and HM peptone B supply nitrogenous and carbonaceous compounds. Yeast extract provides vitamin B complex and dextrose is the fermentable carbohydrate and energy source. Polysorbate 80 supplies fatty acids required for the metabolism of Lactobacilli. Sodium acetate and ammonium citrate inhibit Streptococci, moulds and many other microorganisms.

TABLE 3: COMPOSITION OF MRS BROTH

Ingredients	Amount (Gm\L)
Proteose peptone	10.0
HM Peptone B	10.0
Yeast extract	5.0
Dextrose	20.0
Tween 80	1.0
Ammonium citrate	2.0
Sodium acetate	5.0
Magnesium sulphate	0.1
Dipotassium hydrogen phosphate	2.0
Manganese sulphate	0.05
Agar	12.0
Final pH	6.5±0.2

Magnesium sulphate and manganese sulphate provide essential ions for multiplication of lactobacilli. Phosphates provide good buffering action in the media. Lactobacilli are microaerophilic and generally require layer plates for aerobic cultivation on solid media.

Preparation of MRS Broth: About 67.15 grams of MRS broth was suspended in 1000ml purified / distilled water. It was heated to dissolve the medium completely and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. The broth was subjected to cooling at 45-50°C. The broth was poured into sterilized petriplates. MRS Media shown in **Fig. 2** was obtained from the market manufactured by Titan Biotech Ltd.

FIG. 2: PICTURE OF MRS BROTH

Pelletization: For the preparation of pellet, we require luxuriant colonies of the microorganism. Hence the samples A+, milky mist, goat milk and zebiotic samples were grown separately in MRS broth as shown in **Fig. 3**. One loop full of each colony identified in nutrient agar media was taken and inoculated in MRS broth for 48 hrs. 10ml of the above broth containing of each sample was taken and centrifuged for 10 min at 5000rpm. The supernatant was discarded and the pellet weighing approximately 1 gm was Held using few drops of sterilized deionized water.

FIG. 3: MRS BROTH CONTAINING CULTURES OF EACH SAMPLE

Preparation of Probiotic Milk Chocolate: Initially, milk chocolate was prepared by taking 200gm of milk powder and 2 Chocolate cubes (40gm) was heated until it melts. Then addition of some volume of milk (for solidification) was done. When it reaches the normal temperature, freeze dried bacteria was added. After mixing it was stored in freezer at low temperature for solidification and wrapped around by aluminum foil.

The prepared chocolate obtained was melted in double boiler and allowed to reach a temperature of approximately 37°C. To inoculate probiotics, the pellet equivalent to 10^6 $CFUg^{-1}$ was added to the chocolate mass at 36–37 °C, in the proportion of 1 part of bacterium to 4 parts of chocolate in sterile condition. The combination was poured in sterilized moulds for solidification. The solidified chocolate was then wrapped in aluminium foil in sterile condition.

Viability testing: The lab made milk chocolate of the samples were further dissolved in sterilized distilled water and a loop full was inoculated to the nutrient medium containing petriplates for determining the viability of organisms after incubation by performing evaluation tests.

Evaluation of Probiotic Milk Chocolates: The probiotic milk chocolate was evaluated for various physiological and biochemical characteristics.

Evaluation Test for Bacteria:

A. Color: Creamy

B. Shape: Circular

C. Motility: Nonmotile

D. Gram staining

Motility Test: A very small drop of bacteria suspension was hung from the center of a cover slip into the cavity of a cavity slide. The hanging drop is observed under a microscope using oil-immersion objective. If the bacteria are motile, its cells can be seen to have erratic movement in the surrounding medium. In contrast, if it is non-motile, its cells remain static in the medium without any movement or may show Brownian movement resulting from the bombardment by the

water molecules in the medium, on the bacteria cells. A small drop of the suspension was placed at the center of a cover slip. The broth culture should not be more than 24 hours old, because bacteria may lose their motility, as they grow older. The cavity slide was inverted and placed on the cover slip, in such a way that, the cavity covers the drop. The slide and cover slip are pressed together gently, so that the cavity is sealed. The slide was inverted quickly, such that the drop hangs into the cavity without touching it. The slide was clipped to stage of the microscope. The edge of the drop is focused under oil immersion objective.

Gram Staining: The first step in gram staining is the use of crystal violet dye for the slide's initial staining. The next step, is fixation of dye which involves using iodine to form crystal violet- iodine complex to prevent easy removal of dye. Subsequently, a decolorize, often solvent of ethanol and acetone, is used to remove the dye. The final step in gram staining is to use basic fuchsine stain to give decolorized gram-negative bacteria pink color for easier identification. It is also known as counter stain. Some laboratories use safranin as a counter stain; however, basic fuchsine stains gram-negative organisms moreintensely than safranin.

Biochemical Tests: The various biochemical tests were performed for the identification and confirmation of the bacterial species.

Indoletests: This test demonstrates the ability of certain bacteria to decompose the amino acid tryptophane to indole, which accumulates in the medium. break down the amino acid tryptophan with the release of indole. This is performed by a chain of a number of different intracellular enzymes, a system generally referred to as "tryptophanase". Sterilized test tubes containing 4 ml of tryptophan broth. The test tube were inoculated aseptically with the growth from 18 to 24 hours culture. The test tubes were maintained at 37°C for 24-28 hours. 0.5 ml of Kovac's reagent was added to the broth culture and the presence or absence of ring was observed.

Methylred test: This test is to determine the ability to utilize glucose and convert it to a stable acid like lactic acid, acetic acid or formic acid as the end product. Prior to inoculation, the medium was allowed to equilibrate at room temperature. Organisms was taken from an 18–24-hour pure culture, lightly inoculated the medium. Incubated aerobically at 37 ^{o}C. for 24 hours. After 24 hours of incubation, 1ml of the broth was added to a clean test tube. The test tubes were reincubated for additional 24 hours. 2 to 3 drops of methyl red indicator was added to a liquor and development of red color was examined.

Before inoculation the medium was equilibrated to room temperature. Sterilized test tubes containing 4ml of tryptophan broth was taken. Aseptically the Growth from 18-24 hours culture was inoculated with it.

Vogues Proskauer: It is used to determine if an organism produces acetyl methyl carbinol from glucose fermentation. Prior to inoculation, medium was equilibrated to room temperature. Organisms taken from an 18-24-hour pure culture, lightly inoculate the medium. Incubate aerobically at 37 degrees C. for 24 hours. Following 24 hours of incubation, aliquot 2ml of the broth to a clean test tube. Re-incubate the remaining broth for an additional 24 hours. Add 6 drops of 5% alpha-naphthol, and mix well to aerate. 2 drops of 40% potassium hydroxide was added, and mix well to aerate. A pink-red color was observed at the surface within 30 min. Shake the tube vigorously during this 30-min period.

Catalase test: This test demonstrates the presence of catalase, an enzyme that catalyzes the release of oxygen from hydrogen peroxide (H_2O_2). It is used to differentiate those bacteria that produces an enzyme catalase. Use a loop or sterile wooden stick to transfer a small amount of colony growth in the surface of aclean, dry glass slide. A drop of 3% H_2O_2 was placed in the glass slide and observed for the evolution of oxygen bubbles.

Confirmation Test for Bifidobacterium:

Glucose Fermentation test: It tests an organism ability to ferment the sugar glucose as well as its ability to convert the end products of glycolysis, pyruvate acid into gaseous byproducts. A loop full of sample was added to test tube containing glucose broth to which Yellow color is observed confirms the Bifidobacterium.

Lactose Fermentation test: It tests an organism ability to ferment the sugar lactose as well as its ability to convert the end products of glycolysis, pyruvate acid into gaseous byproducts.

A loop full of sample was added to test tube containing lactose broth to which phenol red indicator is added. Yellow color is observed confirms the Bifidobacterium.

Evaluation of Probiotic Milk Chocolates: Changes in the physicochemical properties of the probiotic milk chocolate during 90 days of storage at 4^{o}C were monitored and compared with the control milk chocolate.

Sensory Evaluation: In terms of appearance color, flavor, texture, and overall acceptability, sensory qualities of control and probiotic-supplemented chocolates were assessed.

Probiotic Viability in Milk Chocolate: The lab made chocolate was added to sterilized distilled water and a loop ful was inoculated to the nutrient medium containing petri plates for determining the viability of lactobacillus species after incubation.

RESULTS AND DISCUSSION: Nutrient medium plates were observed to have isolated bacterial sample from curd. The bacterial sample was further tested for confirmation of lactobacillus and bifidobacterium species.

Identification Tests for Bacterial Species: The isolates of bifido bacterium used in the present study were polymorphic branched rods that occur singly or in chains or clumps and the colonies of bifidobacterium were found convex, round and regular white colonies on agar plates. The isolates of lactobacillus species were found be rod shaped and the colonies of lactobacillus species were found slightly mucoid and creamy in color as shown in **Table 4.**

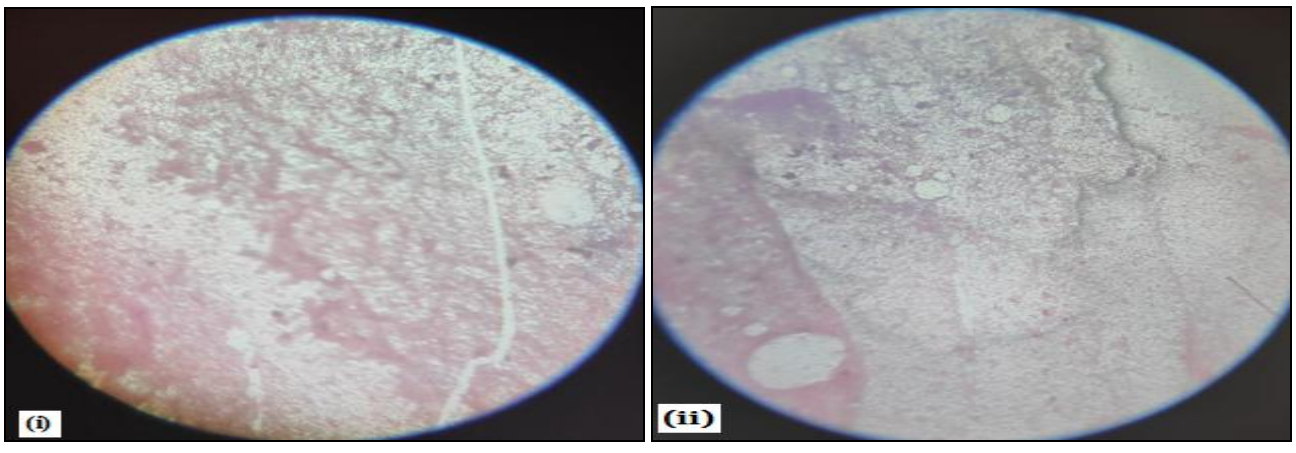
TABLE 4: IDENTIFICATION TEST FOR LACTOBACILLUS AND BIFIDOBACTERIUM

Strain	Morphological characteristics	Cultural characteristics	Biochemical characteristics							
			Glucose	Fructose	Lactose	Methyl Red	Catalyse	Indole	Mv	Citrate
Bifidobacterium	Non motile Gram +ve Rod shaped or coccobacilli	Round Regular white colonies	+	-	+	-	-	-	-	-
Lactobacillus acidophilus	Non motileGram +ve Rod shaped	Circularlarge Creamycolonies	-	-	-	-	-	-	-	-

Gram Staining Test: As shown in **Table 5** gram staining was performed and the entire samples shown positive for gram staining except placebo, indicating that these samples contain gram positive bacteria. The results are shown in **Fig. 4.**

TABLE 5: GRAM STAININ

Samples	A+ curd	Milky mist	Goat milk	Zebiotic capsules	Placebo
Test result	+	+	+	+	-



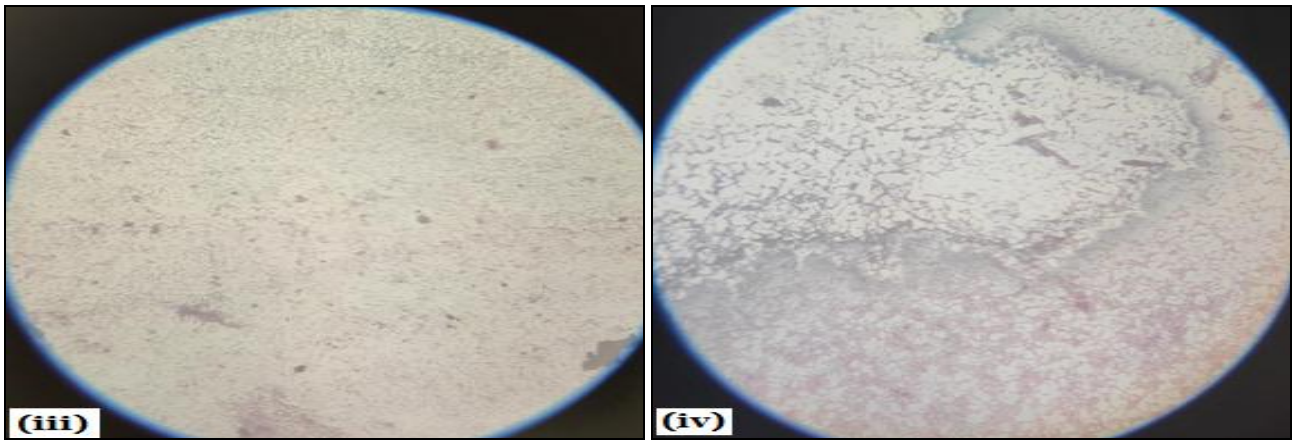


FIG. 4: GRAM STAINING TEST (I) A+ CURD (II) MILKY MIST (III) GOAT MILK (IV) ZEBIOTIC CAPSULES

Motility Test: Motility test was performed to all the samples as shown in **Table 6** and were found non motile. **Fig. 5** shows the pictures motility of different samples. Placebo lack evidence of any organism.

TABLE 6: MOTILITY TEST

Samples	A+ curd	Milky mist	Goat milk	Zebioticcapsules	Placebo
Test result	Non motile	Non motile	Non motile	Non motile	-----

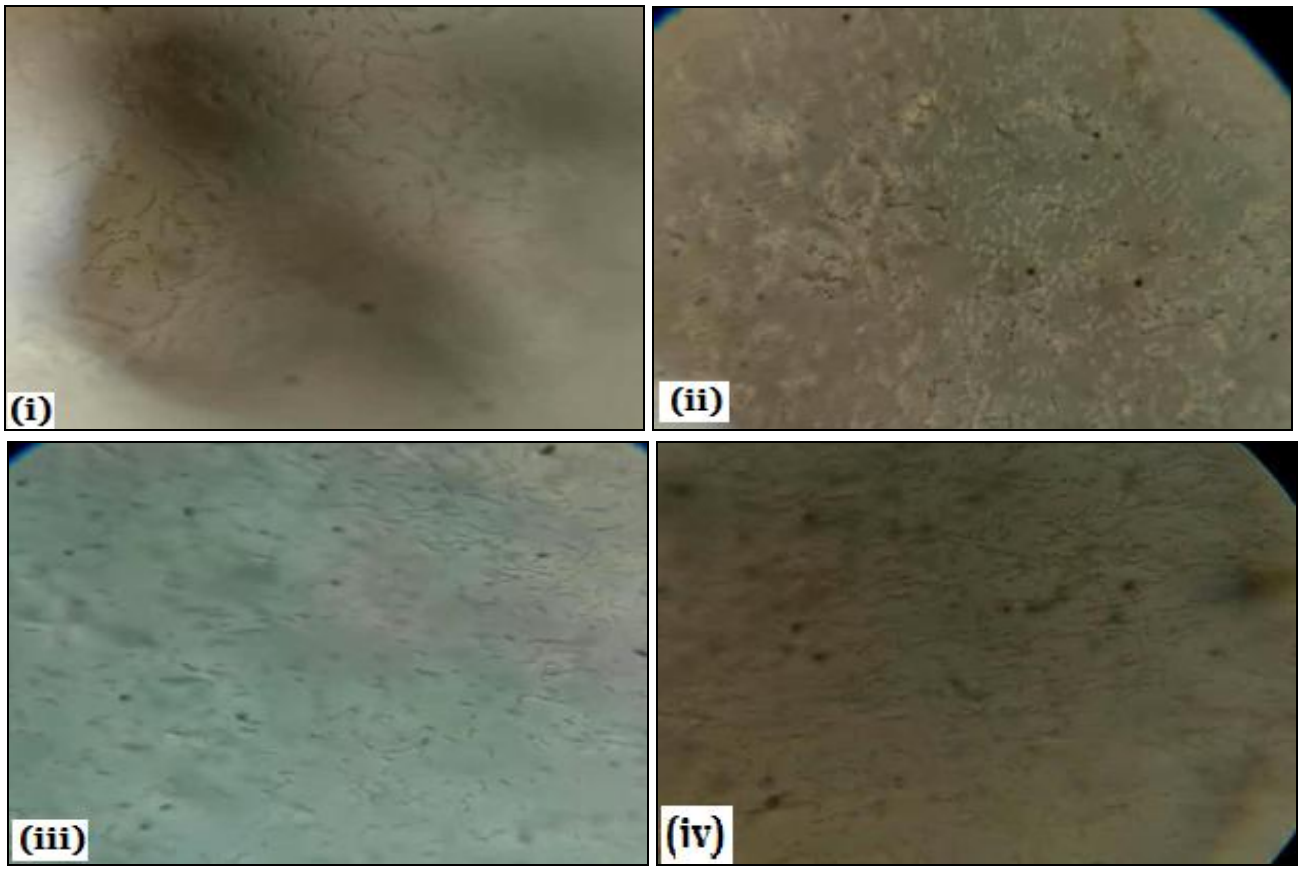


FIG. 5: MOTILITY TEST (I) A+ CURD (II) MILKY MIST (III) GOAT MILK (IV) ZEBIOTIC CAPSULES

Biochemical Tests: Biochemical tests of cultures obtained from milkymist and zebiotic samples shown positive result for glucose and lactose test indicating that these samples contain bifidobacterium and shown negative for A+, goat milk and placebo samples as shown in **Table 7**. All the samples shown indole, methyl red, catalyze test gave negative results.

TABLE 7: BIOCHEMICAL TESTS

Samples	A+ curd	Milky mist	Goat milk	Zebioticcapsules	Placebo
Glucose	-	+	-	+	-
Lactose	-	+	-	+	-
Indole	-	-	-	-	-
Methyl red	-	-	-	-	-
Catalyse	-	-	-	-	-

Evaluation of Probiotic Milk Chocolates:

Probiotic Milk Chocolate Viability: The lab made chocolates were further dissolved in sterilized distilled water and a loopful of all the samples were inoculated to the nutrient agar medium containing petri plates for determining the viability of lactobacillus and bifidobacterium species after incubation for 24 hours **Fig. 6.**

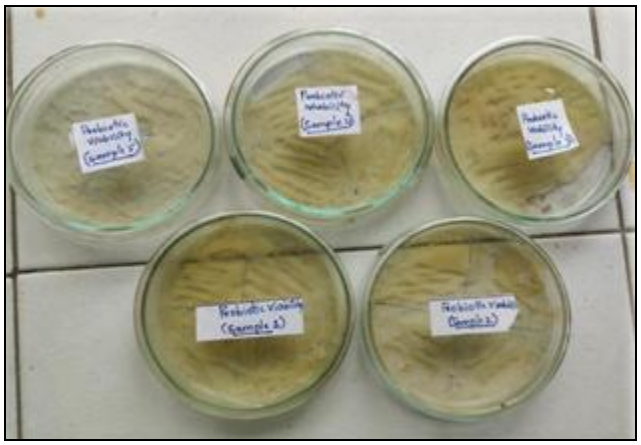


FIG. 6: PROBIOTIC VIABILITY OF CHOOSEN SAMPLE

Antimicrobial Action: The inhibitory test showed that all the samples used in the present study inhibited the growth of selected enteropathogens *i.e E. coli*, *Bacillus subtilis* and *Staphylococcus aureas* except placebo shown in **Table 8**. A+ curd sample inbitory activity was observed against *E. coli* is 25mm, *Bacillus Substilis* is 28mm, *Staphylococcus aureas* is 21mm.

Milky mist curd sample inhibitory activity was observed against *E. coli* is 30mm, *Bacillus Substilis* is 32mm, *Staphylococcus aureas* is 29mm.

Goat milk sample inhibitory activity was observed against *E. coli* is 28mm, *Bacillus Substilis* is 30mm, *Staphylococcus aureas* is 24mm.

Zebiotic sample inbitory activity was observed against *E. coli* is 33mm, *Bacillus Substilis* is 34mm, *Staphylococcus aureas* is 32mm. No inbitory activity was observed against *E. coli*, *Bacillus Substilis* and *Staphylococcus aureas* by placebo.

TABLE 8: ANTIMICROBIAL ACTION

Samples	Zone of inhibition		
	E. coli	***Bacillus subtilis***	***Staphylococcus aureas***
A+ curd	25mm	28mm	21mm
Milky mist	30mm	32mm	29mm
Goat milk	28mm	30mm	24mm
Zebiotic capsules	33mm	34mm	32mm
Placebo	- - - -	- - - -	- - - -

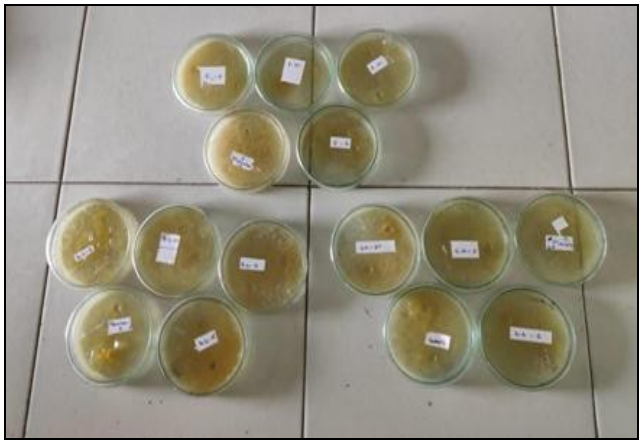


FIG. 7: ANTIMICROBIAL ACTIVITY OF ALL SAMPLES

It was observed from **Table 8** that all the probiotic samples selected have shown antibacterial effect except placebo shown in **Fig. 7**. The A+ curd sample contains Lactobacillus species alone. It was shown the lowest inhibitory activity when compared to milky mist curd sample, goat milk sample and Zebiotic sample.

The goat milk sample has shown inhibitory effect more than A+ curd sample because it has abundant presence of Lactobacillus species alone. The inhibitory activity of milky mist curd samples was found to be higher than A+ curd sample and goat

milk sample as it contains two probiotic species Lactobacillus and Bifidobacterium. We observed additive action with this sample. Zebiotic sample have shown highest inhibitory effect as it contains 4 probiotic species.

Therefore it is proven that all the probiotic samples selected have significant activity aganists enteropathogens i.e; *E. coil, Staphylococcus aureus* and *Bacillus subtilis*. Hence the formulation of these Samples as probiotic milk chocolate is an novel idea in therapeutics for treating gastrointestinal disorders.

CONCLUSION: Chocolate contains milk products thus acts as a carrier for probiotic and it contains natural antioxidants. In the current study samples containing Lactobacillus, Bifidobacterium and chosen and evaluated for physicochemical, cultural properties and viability. The concentration was maintained at 10^6 CFU/gm. The enteropathogens used in the present study are the causal organisms of various diseases like dysentery, urinary tract infections and food poisoning. It was observed that the isolate of Bifidobacterium and Lactobacillus acidophilus inhibits the growth of these enteropathogens. Hence, it could be concluded that these species can be used to prevent and treat illness caused by these pathogens. The use of these species in the treatment of many forms of gastrointestinal diseases appears especially promising. Coupled with this the alarming rise in antibiotic resistance and the emergence of many multidrug resistant strains emphasize the need for novel thinking and approaches for the development of alternative therapeutics for the treatment of gastrointestinal disorders with advancement in technologies and further refinements and developments in new techniques. Research in this area will continue to provide novel bio therapeutics and therapeutic targets as well as novel probiotic strains for the treatment and prevention of gastro intestinal disorders. The results indicate inclusion of probiotic in chocolate is an excelled solution to protect from different diseases.

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