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# Study of normal and pathogenic bacteria and yeasts in curd samples

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ABSTRACT

Probiotics are very important for our daily life and as food. However, they must be safe to consume. There can be many bacterial contaminants present in curd bought from the market, which can cause infections. We studied curd samples and found many such pathogens as well as Acid fast bacilli. Hence, one should be cautious while consuming curd bought from market. This is an important but neglected area of public health research.



## 1. Introduction

Probiotics are important components of our diet and are helpful for our health in many ways. However they may sometimes also contain harmful bacteria and fungi. Marketed probiotic products may contain many unspecified microbes of their components. One should know precisely the definitions of probiotics and their beneficial effects which are listed as follows.

### *1.1. Prebiotics*

The term "biotics" refers to prebiotics, probiotics, synbiotics and postbiotics, that have an impact on homeostasis. Prebiotics are the food for the microorganism (bacteria) in the microbiota. The common prebiotics are inulin and fructooligosaccharides, which helps to stimulate multiplication of Bifidobacterium spp. and Lactobaccillus spp.[1] One of the most effective public health methods for preventing infections is to strengthen immunity by functional nutrition options like prebiotics and probiotics.[1]

### *1.2. Probiotics*

'Probiotics is a new term which means for life'. It's a Greek word.

The term probiotics refers to live microorganisms in low doses which can exert favourable function on the host microbiota and health.[2] Probiotic bacteria produce short chain fatty acids or SCFA and many other substances which can affect immune functions of the host like the populations of regulatory T- cells.[1] Probiotics are beneficial bacteria that can be found in gut and are beneficial for human body when consumed in small to moderate amounts.[3] The probiotic microorganisms comprise bacteria like Lactobacillus, Bifidobacterium, Bacillus, Pediococcus, Lactococcus, and yeasts of genus Saccharomyces, which confer many health benefits to the host. Probiotics are live microorganisms that helps to treat various pathologies including Inflammatory Bowel Disease (IBD) (Ulcerative colitis and Crohn's disease), Constipation, Irritable Bowel Syndrome (IBS), Diarrhoea (acute and antibiotic related), Diabetes Mellitus (DM), Hypertension (HTN). It also helps to treat in allergic conditions.[2]

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Probiotics can be found in dairy products like milk, curd and yogurt, as well as non-dairy products like Kimchi salad, Sauerkraut etc. Overuse and prolonged use of oral antibiotics like beta-lactams and Clindamycin can lead to depletion of beneficial gut microbes producing antibiotic associated diarrhoea, which can be ameliorated by the use of probiotics. Dairy products are especially contemplated as perfect transporters for delivering probiotic bacteria into the human gastrointestinal tract (GIT).

In recent research there is increasing evidence in favour of the claims of the beneficial effects attributed to probiotics, including improvement of intestinal health by formation of short chain fatty acids (SCFAs), enhancement of the immune response, reduction of serum cholesterol, improvement of lactose metabolism so that it directly helps to alleviation of lactose intolerance, cancer prevention (anti-carcinogenic), anti-microbial, anti-ageing, anti-diabetes, anti-biofilm effects.[4,5]

### *1.3. Effect of probiotics in human health*

The natural defence mechanism in the body can be boosted by probiotics.[6] Probiotic food are increasingly being used in treatment and also minimise the intensity of the disease in various human health related problems such as food allergy, atopic dermatitis, acute gastroenteritis, colon cancer, arthritis etc.[7,8]

### *1.4. Synthesis and bioavailability of nutrients*

Probiotic microbiota intensify nutrients availability and helps to digest easily during fermentation and absorption of fermented food. Riboflavin (Vit B2), Niacin (vit B3), folic acid (vit B9), are well present in fermented dairy product.[9,10] Enzymatic hydrolysis of bacteria can be support in protein and fat digestion which further leads to enhanced the bioavailability of nutrients and also increased the production of functional components.[11]

According to recent studies, probiotics has a role in therapeutic management, like in treatment of rheumatoid arthritis, administration of probiotics capsule may be beneficial.[12] Probiotics are seems effective particularly in food allergy and atopic dermatitis, few bacterial strains had been tested to ensure the efficiency of effectiveness in prevention and treatment as well. So, in atopic eczema, it is observed that administration of Lactobacillus rhamnosus GG and B. Lactis decrease the intensity of eczema in infants.[13,14] Probiotics are found useful in treatment of inflammation, by regulating the inflammatory mediators like cytokines (immunomodulating agent).[13]

Probiotic diary products like yoghurt are reported to be anticarcinogenic and antagonist against many pathogens.[15] Probiotics contain active live microorganisms (bacteria) such as Lactobacilli, Bifidobacteria or Lactococci.[16] According to recent studies, probiotics may also contain some pathogenic bacteria such as Streptococcus spp., Leuconostoc spp., Pediococcus spp., Bacillus spp.[17] Unregulated use of probiotics, on the other hand, can lead to serious health issues.[18] According to previous research it was observed that the claimed beneficial microorganisms in probiotics may not actually be present. According to examination of probiotics product it has been proved that the microbial species do not always correlate or corroborate with the information mentioned in the product levels.[18–20]

Probiotic food affects health and well being in a favourable way, because it is an assembly of live microbial cell. In recent studies, it also found that probiotics are used as preventive and therapeutic measure. As for example, in some pathological condition probiotics live microorganism is consumed for have a positive/constructive effect in prevention and treatment as well. As probiotics is used in gastrointestinal diseases such as (IBD, IBS) it can be contemplate that probiotics is a therapeutic agent.[21] Lactobacillus, Bifidobacterium and Streptococcus (S. thermophilus) are the most preferably used stains of lactic acid bacteria (LAB) in probiotics, in this first two are known to endure gastric acid, bile acid, and pancreatic enzyme, to adhere to colonic mucosa and willingly/readily colonize the intestinal tract.[21]

As probiotics is a live microorganisms it evolve a synergistic/complementary relationship with the host when administrated in the proper doses. The lactic acid bacteria (LAB) present in the gut are generally consider as probiotics, this includes Lactobacillus rhamnosus, L. casei, L. acidophilus, and Bifidobacterium lactis, among others. The bacteria procure all essential nutrients as well as energy to sustain/survive in the gut, from the food consumed by the human and in return it helps to keep alive the gut microbiota. According to research it can contemplate that probiotics will promote immune function, improve mental health, and also protect against cognitive impairment.[22]

Children those who are having lactose intolerance and taking antibiotics during acute infectious diarrhoea, administration/consumption of probiotic tablets is associated with a reduced severity of diarrhoea and intervals of defecation, it also helps to improve proper lactose assimilation and calcium absorption as well by hydrolysis of milk lactose through probiotic strains. Some probiotics can inhibit the growth of Helicobacter pylori which can cause ulcer in the stomach and may develop cancer if untreated for several years.[4,22]

Research suggest that the incidence of yeast infection can be reduced by consumption of yogurt. The risk of upper respiratory infection in adults and cold, flu in children can be decreased by consumption of probiotics. Obesity and type II diabetes can be occur due to alteration of gut microbiota. Probiotics has a role in the prevention of obesity and diabetes but still more research are needed to prove these findings. In some studies, probiotics may linked with

mental health and also reduced the stress and anxiety but many more research are needed to prove it.[22]

The role of various probiotics in various health conditions is summarized in the table below:

### *1.5. Synbiotics*

Synbiotics refer to the functional combination of prebiotics and probiotics. Indeed synbiotic combination are considered to have more beneficial effect on human health than prebiotics or probiotics alone.[23]

### *1.6. Postbiotics*

Postbiotics are the bioactive substance that are released by or produced through the metabolic action of microorganisms, which has a beneficial effect on the host.[24] The metabolic by product of live probiotic bacteria include various postbiotics molecule such as vitamins, organic acids, short chain fatty acid, secreted proteins/peptides, neurotransmitter, bacteriocins, secreted biosurfactant, amino acids, flavonoids derived postbiotics (desaminotyrosine, equol daidzein, daidzein, norathyriol), terpenoids derived postbiotics (genipin, paeoniflorin, paeonilactone glycosides, paeonimetabolin I, II, III), phenolic-derived postbiotics (equol, urolithins, valerolactones, enterolactone, enterodiol, 8-prenylnaringenin) etc.[25,26]

Pathogenic bacteria like Streptococcus spp., Leuconostoc spp., Pediococcus spp., Bacillus spp. can also be present in curd and other probiotics.[17] Sometimes diarrhoeagenic Escherichia coli may also be present. It may also be so that some undisclosed or unwanted microbes are present in commercial or medicinal probiotics available in the market. Hence it is important to know the prevalence of normal as well as disease-causing bacteria and yeasts in various home-made and commercially available probiotic preparations. This can be correlated with pH also. As far as we know such a study has not been carried out in this part of the country. So our study is important with that respect.

So, probiotics and postbiotics are important for maintaining health. Probiotics are useful for treatment of Irritable Bowel Disease and can also be related with good sleep. Probiotic bacteria also maintain intestinal health. Probiotics can be defined as live microorganisms, which when administered in adequate amounts, confer a health benefit on the host.[27] This concept of administering microorganisms in order to have a positive health benefit began over a century ago when Elie Metchnikoff postulated that health could be enhanced, and also old age could be delayed, by manipulating the intestinal microbiome with host-friendly bacteria found in yogurt.[28]

The genus Lactobacillus includes various Gram positive facultative anaerobic or microaerophilic rod-shaped bacteria. They are a major component of the lactic acid bacteria (LAB) group (like Lactobacillus, Lactococcus, Enterococcus, Oenococcus, Pediococcus, Streptococcus and Leuconostoc species) which can convert hexose sugars to lactic acid, thus producing an acid environment which inhibits the growth of several strains of harmful bacteria.[29]

Yeasts can also form part of probiotics. Saccharomyces boulardii (S. boulardii) as probiotics, can be effective in gastrointestinal pathologies such as inflammatory bowel disease (IBD) and bacterial- or enterotoxin-mediated diarrhoea and inflammation.

However, expired yogurt or curd can cause intestinal distress and diarrhoea in pregnant women, young children, the elderly and people with a compromised immune system such as those with HIV.[30] In a study in Brazil, in curd cheese Staphylococcus aureus and Escherichia coli could be found.[31] In another study in Egypt, Staphylococcus aureus was found in some yogurt samples and its enterotoxin could also be detected in those samples by PCR.[32]

Several outbreaks have been reported of infection due to Enterohemorrhagic Escherichia coli 0157: H7 caused due to eating curd from unpasteurized milk.[33]

People with lactose intolerance should not be given milk-based probiotics. Because microbes used as probiotics already exist naturally in our body, probiotic foods and supplements are generally safe. However, hey may trigger allergic reactions, and may also cause mild stomach upset, diarrhoea, or flatulence and bloating for the first few days after starting to take them.[34]

**Fig. 1:** Picture of curd

Hence here we aimed to study the normal and pathogenic bacteria and yeasts present in various probiotics like natural foods and commercially marketed probiotics, by.

1. Collecting Samples by proper and aseptic technique.

**Table 1:** Probiotics in various conditions

| Probiotic bacteria or yeast strain | Health condition where useful | Remarks |
|---|---|---|
| Bacillus coagulans | Antibiotic–associated diarrhoea | Rarely used |
| Bacillus subtilis | Necrotizing enterocolitis Radiation-associated diarrhoea | Not commonly seen in marketed probiotics |
| Lactobacillus reuteri | Antibiotic–associated diarrhoea C. difficile infection Colic Functional abdominal pain Helicobacter pylori infection Necrotizing enterocolitis Traveler's diarrhoea Ulcerative colitis | Commonly used |
| Lactobacillus casei | Irritable bowel syndrome | Commonly used |
| Lactobacillus acidophilus | Acute pancreatitis Antibiotic–associated diarrhoea C.difficile-associated diarrhoea C. difficile infection Crohn disease Functional abdominal pain Helicobacter pylori infection Hepatic encephalopathy Irritable bowel syndrome NAFLD/NASH Necrotizing enterocolitis Radiation-associated diarrhoea Traveler's diarrhoea Ulcerative colitis | Commonly used |
| Lactobacillus plantarum | Acute pancreatitis Antibiotic–associated diarrhoea C.difficile-associated diarrhoea C. difficile infection Crohn disease Functional abdominal pain Hepatic encephalopathy Irritable bowel syndrome NAFLD/NASH Necrotizing enterocolitis Radiation-associated diarrhoea Ulcerative colitis | Commonly seen |
| Lactobacillus rhamnosus | Antibiotic–associated diarrhoea C. difficile infection Helicobacter pylori infection Irritable bowel syndrome NAFLD/NASH Necrotizing enterocolitis Radiation-associated diarrhoea Ulcerative colitis | Commonly used |
| Lactobacillus rhamnosus GG | Acute infection diarrhoea Antibiotic–associated diarrhoea C.difficile-associated diarrhoea C. difficile infection Chemotherapy-associated diarrhoea Crohn disease Functional abdominal pain Helicobacter pylori infection Hepatic encephalopathy Irritable bowel syndrome NAFLD/NASH Necrotizing enterocolitis Traveler's diarrhoea Ulcerative colitis | Very common strain |
| Lactococcus lactis | Irritable bowel syndrome | Very common strain |
| Streptococcus thermophilus | Acute pancreatitis Antibiotic–associated diarrhoea C.difficile-associated diarrhoea C. difficile infection Crohn disease Functional abdominal pain Functional constipation Helicobacter pylori infection Hepatic encephalopathy Irritable bowel syndrome NAFLD/NASH Radiation-associated diarrhoea Traveler's diarrhoea Ulcerative colitis | Rarely found |
| Saccharomyces boulardii | Acute infection diarrhoea Antibiotic–associated diarrhoea C. difficile-associated diarrhoea C. difficile infection Crohn disease | Commonly found in both curd and medicinal probiotics |
| Bacillus cereus | Necrotizing enterocolitis | Not common and sometimes pathogen also |
| Enterococcus faecalis | Acute pancreatitis Irritable bowel syndrome Necrotizing enterocolitis | Somewhat common |

2. Transporting samples timely to the laboratory and processing of the samples in laboratory.
3. Identifying the normal and pathogenic bacteria and fungi present.

## 2. Materials and Methods

### *2.1. Time of study*

The study was carried out from September 2022 to January 2023.

### *2.2. Place of study*

Department of Microbiology, BN campus, All India Institute of Hygiene and Public Health, Kolkata.

### *2.3. Type of study*

Laboratory based observational study.

### *2.4. Sample size*

One hundred and two probiotic samples (102) were tested by us, out of which 52 were curd samples. This samples size was calculated by method of convenience. Here only data of the 52 curd samples are shown. There were 35 loose curd

samples bought from sweet shops, 14 packed branded curd from shops, and 3 in-house made yogurts/curd.

### 2.5. Methodology proper

Samples were collected from

(A). Kolkata, (B). Domjur, Howrah, (C). Bidhannagar, North 24 Parganas district. (D). Durgapur, West Barddhaman district. (E). Sodepur, North 24 Parganas.

The sites of sample collection are shown in Figure 2.

Samples were collected in sterile universal container or if packed, then in packet or in case of curd from sweet shop, earthen containers. In-house samples were made in sterile universal containers. The date of packaging, expiry date and brand name were all noted. Then samples were transported to laboratory in ice-pack or within 4 hours of collection. The pH of the samples were also noted. Then the samples were processed for bacteria and yeasts. Samples were weighed, and specific weight or volume were inoculated on the following media:

1. Mac Conkey agar with neutral red as pH indicator (Peptone, Neutral red, agar agar, Lactose, Sodium taurocholate, deionized water) for bacteria.
2. Sabouraud's dextrose agar plate (pH 5.6-6) (glucose – 2gm, Peptone 2 grams, Agar agar 2 grams, deionized water 100 ml) for fungi.
3. Robertson's cooked meat medium (RCM) for culturing anaerobes, made as per manufacturer's instructions.
4. Tomato juice agar for lactobacilli ( formula appended below).
5. For lactobacilli, plates were kept in microaerophilic incubation using glass candle jar and white burning candle.

Simultaneously, Gram's stain, Albert's stain and conventional Ziehl-Neelson's stain were done from samples directly to detect bacteria, bacteria with metachromatic granules and acid fast bacilli, respectively.

After inoculating the samples, plates were incubated at 37 Degree C overnight, i.e. for 12 to 16 hours, and then observed for colonies. Tomato juice agar was incubated in microaerophilic conditions in candle jar. RCM tubes were observed after 48 hours of incubation and Gram stain were done from RCM to detect spore bearing anaerobic bacilli. Colonies on MacConkey agar and Tomato juice agar were identified by colony morphology, Gram stain, Albert's stain for bacteria or yeasts with metachromatic granules and also ZN (Ziehl-Neelson) stain if required. Lactobacilli were identified by Gram stain morphology (Gram positive uniformly staining bacilli) and small punctuate or pitted colonies, or sometimes translucent colonies on Tomato juice agar, with inherent resistance against Vancomycin and variable resistance against Nitrofurantoin. Biochemical tests like motility, Citrate utilization, acid and $H_2S$ on TSI agar were also done from colonies of Gram negative bacteria, and from Gram positive colonies, standard biochemicals like Catalase (with 3% $H_2O_2$), coagulase and oxidase were done. Lactococcus was identified by magenta colonies on MacConkey agar and no growth at 44 degree C. Enterococcus spp. were identified by magenta colonies on MAC Conkey Agar, positive growth in 6.5 % NaCl, positive aesculin hydrolysis and positive growth at 44 degree C. Bacillus spp. and E. coli were identified by standard biochemical tests and molds by microscopic morphology on Lactophenol cotton blue mount from colonies on SDA.

Yeasts were identified by Gram stain, Lactophenol cotton blue mount, Germ tube test, growth on SDA at 44 Degree C and also sugar fermentation tests.

For bacteria, their count per ml of sample were calculated.

Hence the data were collected and analyzed. The prevalence of bacteria and yeasts in probiotics were assessed. Recipe of tomato juice agar is given in table 2 below.

**Table 2:** Formula of modified tomato juice agar

| Ingredients | Amounts |
|---|---|
| Tomato juice concentrate | 2gm |
| Peptone | 1gm |
| Milk powder | 0.5gm |
| Whey powder | 0.5gm |
| Agar agar | 1.3gm |

pH of tomato juice agar: 5.0

### 2.6. Inclusion criteria

Simple yogurt or curd and probiotic tablets from medical stores were selected for the study. Curd made in the laboratory or in-house using branded curd sample as starter culture and milk (first incubating at room temperature for 2 hours and then refrigerating overnight), were also taken. However they were within date of expiry.

Exclusion criteria: Sweet curd samples were not considered.

## 3. Results

Hence at the end of the study, we were able to assess the presence of both beneficial and harmful bacteria and yeasts in curd samples, which is very important from public health point of view. This is because probiotics should be safe to consume**.** The correlation of the microbes isolated with pH of the curd samples were also tested.

### 3.1. How many curd samples tested

A total of 52 curd samples (from shop: 49, homemade: 3) were tested. All the surd samples grew bacteria.


SIKKIM
BHUTAN
NEPAL
ASSAM
MEGHALAYA
BIHAR
JHARKHAND
ODISHA
BANGLADESH
West Bengal
BAY OF BENGAL
WEST BENGAL
Country Boundary
State Boundary
National Highway
Major Road
Railway
Rivers
State Capital
Cities
Airport
National Park
Mt. Peak
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Projection - Lambert Tangent
-Durgapur.
-Kolkata
- Sodepur.
-Domjur
- Bidhannagar

**Fig. 2:**

**Fig. 3:** Bacillus colonies

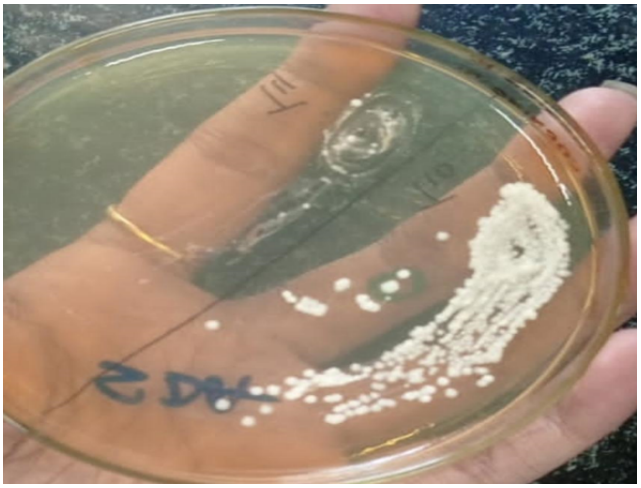

**Fig. 6:** Candida colonies from curd samples

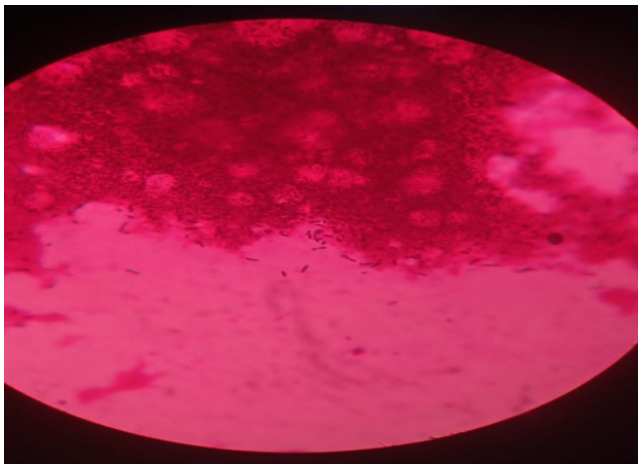

**Fig. 4:** Lactobacillus on Gram stain

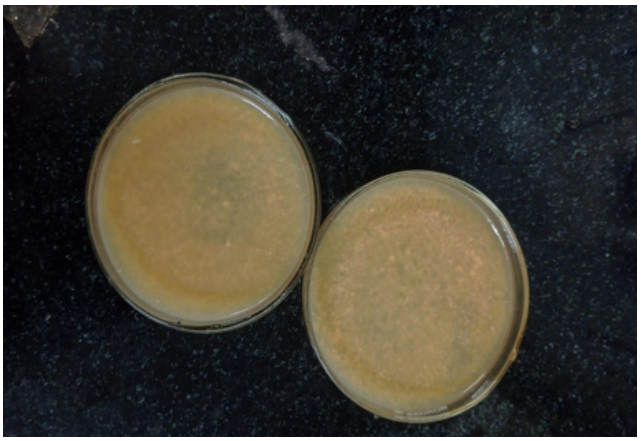

**Fig. 7:** Tomato juice agar

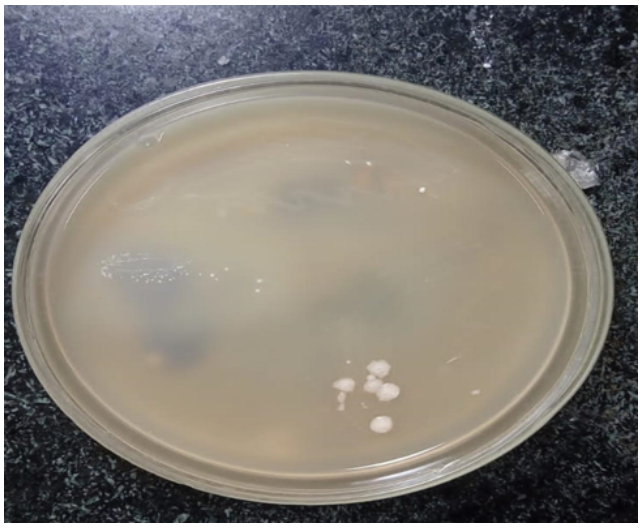

**Fig. 5:** Lactobacillus colonies on Tomato Juice agar

Most common pathogenic bacterium in curd samples was Escherichia coli, followed by Enterococcus and Staphylococcus spp., Candida kefyr and Wickerhamomyces anomalus (earlier Pichia anomala) were also commonly found as pathogens in curd samples.

The commonest probiotic strains found were Lactococcus spp, Lactobacillus spp. and Saccharomyces spp.(yeast).

Out of the 52 curd samples processed, 7 were found to be positive for acid fast bacilli using conventional ZN stain, Those bacilli were thin and short, and may well be Mycobacterium bovis. Six (6) of these AFB-containing samples were loosely sold curd samples and only one was packed curd.

In RCM few curd samples also showed anaerobic bacteria like Peptostreptococcus spp. and Bacteroides spp., which can be pathogenic.

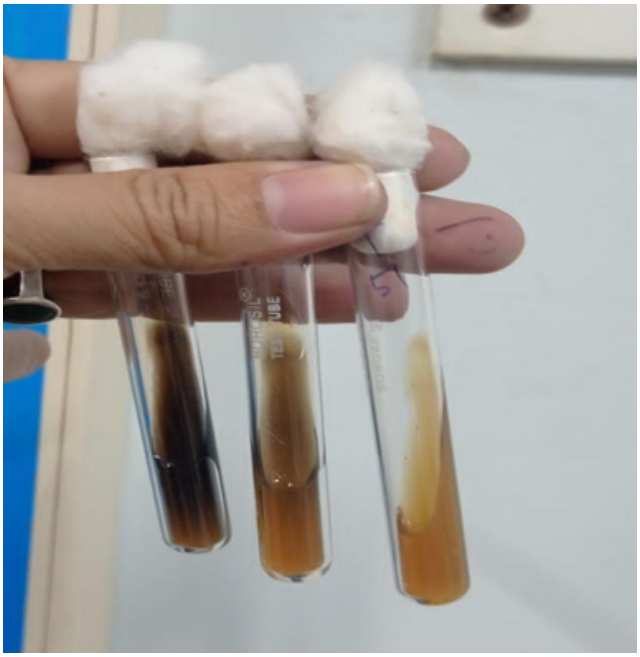

**Fig. 8:** Aesculin hydrolysis in Enterococcus fecalis (extreme left tube)

### *3.2. Correlation with pH*

All pathogens like Proteus spp., Candida and Staphylococcus were almost always found at pH 4 or lesser. Healthy bacteria like Lactococcus and Lactobacillus spp. were identified most commonly in pH more than or equal to 4. Non fermenters like Stenotrophomonas maltophilia were found in pH range 4 to 6. Fungi like Wickerhamomyces anomalus and Rhizopus arrhizus were seen in curd pH more than 5.

## 4. Discussion

Hence at the end of the study we were able to assess the burden of normal as well as pathogenic bacteria and fungi in the probiotics preparations. We found that it is not always safe to consume curd from the market as loose curd can contain many harmful bacteria like Escherichia coli and even molds. Also it may contain acid fast bacilli, most probably Mycobacterium bovis that can cause gut infection. This may come from unpasteurized milk used for preparing the curd. Also we found some anaerobes in loose curd samples, that may be significant from public health viewpoint. This type of study has not been done previously. Hence this findings are very important from the public health view point. Probiotics are now being used for treatment of insomnia and dental diseases also. Also, many probiotics can contain more than one type of microorganisms. Our study was also helpful for finding that. Also, we found out a new medium, modified Tomato juice agar which successfully grew Lactobacilli. Jome made curd had no such pathogens and hence can be had safely. It needs to be seen whether marketed curd is made from Pasteurized milk. Bacterial presence and correlation with pH is also a new idea that can be employed for food science. This can be employed further for food microbiology.

## 5. Conclusion

Hence this study may help us in ascertaining the burden of normal and pathogenic bacteria in probiotics and this will give a new direction and perspective to nutrition and public health research. A new formulation of Tomato juice agar was also tested, and established, which can create a new dimension to public health research and food microbiology. These aspects are important but somewhat neglected. As far as we know, this type of study has not been carried out earlier. So more such studies are needed in this interrelated field of nutrition, infection and public health.

## 6. Abbreviation

Difficile: Clostridium difficile, NAFLD: Non-alcoholic Fatty Liver disease, NASH: Non-alcoholic steatohepatitis.

## 7. Source of Funding

None.

## 8. Conflicts of interest

There are no conflicts of interest.

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