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Isolation and Characterization of Lactobacillus from Curd and Its Application in Food Industry

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Abstract: ***The isolated and identified bacteria from curd sample are of Lactobacillus sp, The bacteria of this spp. are lactic acid producers [1]. Probiotics benefit humans by improving the gut micro biota balance, stimulating immune system and also has antibacterial activities. The lyophilized form of Lactobacillus sp is added to chocolate in two varieties thus probiotic milk chocolate and probiotic dark chocolate are made [2]. The chocolate containing lyophilized Lactobacillus spp. may help directly for ehancing resistance against intestinal pathogens and in the prevention of diseases [3].***

Keywords: Lactobacillus, Curd, Probiotics agents, Evaluation, Immune system, **Antibacterial Activity, Chocolate,** ***Milk chocolate, Dark chocolate, Natural ingredients, Lyophilized lactobacillus spp, Prevention of diseases.***

I. INTRODUCTION

The Lactobacillus is a species of bacteria with many different types. These probiotic bacteria routinely live in our body systems without causing disease. Lactobacillus, which is used for the treatment of diarrhoea, can be found in some fermented foods, including cheese, beer, yoghurt, cocoa, and animal feed. The Lac-to bacillus is a genus of Gram-positive, non-sporing, non-respiring cocci or rods, and can form spirals or spheres under certain conditions. All their energy is obtained by converting glucose into lactic acid during the fermentation of pure lactic acid.[4]

The antagonistic activity of such bacteria is known to inhibit a large number of enteric and urinary pathogenic bacteria (Hutt et al., 2006). Lactic acid bacteria including Lactobacillus, leuconostoc, Lactococcus, pediococcus and Bifidobacterium are found throughout the gastrointestinal tract. Lactobacillus and Bifidobacterium spp. are prominent members of the intestinal flora and are the commonly studied probiotics bacteria. They cause reduced lactose intolerance alleviation of some diarrhoeas, lowered blood cholesterol, increased immune response and prevention of cancer.[5] The aim of this study is to identify L. acidophilus using gram stain, biochemical tests, scanning electron microscopy and automated system for rapid identification of bacteria (BioLog identification system) and optimize the maximum growth of the bacteria using different inoculation methods (pour plate and spread plate method) and cultivation conditions (aerobic and anaerobic conditions).[6] New technology facilitating the supplement of probiotic [lactic acid bacteria] in confectionery will be a health promoting ingredient and will be capable in reducing civilisation disorder.

The present scenario use probiotics to design functional foods to improve the properties of gut microflora and hence a positive effect on human health. The lactic acid producing bacteria which is present in curd was identified to be a probiotic cell. These strains are believed to be beneficial for gastrointestinal track and immune system, reduction of blood cholesterol and apparent anticancer activity.[7] The term bacteriocin was used as a general term for highly specific antibacterial proteins of the colicin type, characterized by lethal biosynthesis, intraspecific activity and absorption to specific receptors and the bacteriocin produced by Lactobacillus fit closely to the classical colicin mode[8] . There are very scanty reports on production of FA using microorganism. In present work, we emphasize on using Lactobacillus spp.[9] Lactobacilli preserve the natural balance and maintenance of natural stability of micro flora present in the Intestine of chickens. They reduce the body and serum fat contents and develop the better poultry performance.

They can also be used in the form of supplements as an alternative for Antibiotics[10] . Valuable effects deliberated by Lactobacilli include inhibition of pathogenic organisms such as Salmonella, Shigella and Helicobacter. Furthermore, Lactobacilli have been associated with various other health Benefits e.g., enhanced immune response and lactose intolerance reduction A beneficial role for Lactobacilli has also been obscure in colon cancer.[10] Lactobacilli boost-up the immune system and treat Cancer, Canker sores, Fever blisters, Hives, General Digestion problems. necrotizing Enter Colitis (NEC) in babies born prematurely. High cholesterol, sensitivity to milk (lactose-intolerance), Common cold, preventing infections in people on ventilators, Lyme disease [10]

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II. MATERIALS AND METHODS

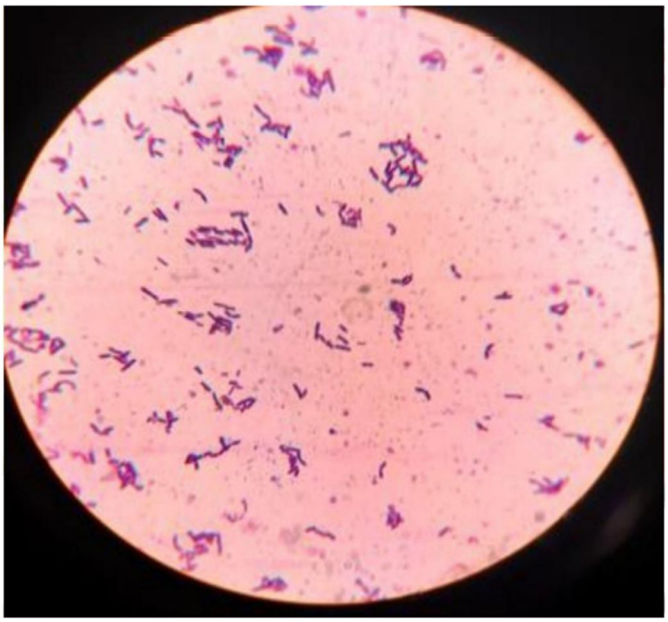
Isolation of Lactobacillus spp. Curd is the best source for lactobacillus spp. Among the other dairy products such as milk, buttermilk etc. Curd is taken in sterilized flask. Under the aseptic conditions curd was serially diluted from 10-2to 10 -5from this dilutions 10-4are selected. Spread plate technique further with streak plate technique is done on MRS medium. They are incubated in incubator 370C which is optimum temperature for Lactobacillus broth. Incubation at 370C for 24 hrs. Broth after 24-48 hrs. shown Lactobacillus species growth and these species for 24 hours. After the period of incubation, the specific isolated colonies were grown. Colony characterization is done for this colonies found to be Lactobacillus species. one colony shows 100% resemblance with Lactobacillus acidophilus. The isolated colony formed on the MRS agar plates was identified using gram stain, biochemical tests. The identification was performed according to Berge's manual of determinative of bacteriology. The culture was kept in MRS agar slant and stored at 4 0C for long term storage.[1]

1) *Isolation of Lactobacillus spp. FROM CURD:* Curd was taken in a sterilized flask. Under the aseptic conditions curd was serially diluted from 10-2to 10-6. Spread plate technique was done on MRS medium. The plates were incubated at 37°C for 24hours. After incubation growth was observed and they were sub cultured for further analysis.[2]
2) *Identification of The Isolated Bacterial Strain:* The isolated colony of bacteria from curd sample was identified by observing its morphology, cultural characteristics and biochemical characteristics. Gram staining, endospore staining, Motility test, catalase test, Oxidase test, Indole test, methyl red test, Voges-Proskauer test, Citrate utilization test, urease test, starch hydrolysis test, carbohydrate fermentation test, NaCl tolerance test, Phenol tolerance test, Milk coagulation assay, Lactose utilization test, antibiotic sensitivity test and antibacterial activity test were performed to identify the bacteria.[2]
3) *Carbohydrate Fermentation Test:* The isolated bacteria were tested for the fermentation of sugars such as glucose, lactose, sucrose and mannitol. The organism was inoculated into carbohydrate fermentation broth and incubated at 37°C for 24 hours.[2]
4) *NACL Tolerance Test:* Fresh overnight culture of Lactobacillus spp.was prepared. This was then inoculated into MRS broth with 4%Nacl concentration and incubated at 37°C for 24 hours. After incubation the turbidity of the culture was measured at 620nm in spectrophotometer.[2]
5) *Phenol Tolerance Test:* Fresh overnight culture of Lactobacillus spp was inoculated into MRS broth with 0.4%phenol concentration and incubated at 37°C for 24 hours. The turbidity of the culture was measured at 620nm in spectrophotometer after Incubation.[2]
6) *Milk Coagulation Assay:* For milk coagulation assay skim milk was taken. Skim milk was prepared by boiling buffalo milk in a non stick pan for 8-10minutes. Cool it for at least 2-3 hours and kept in refrigerator for at least 10-12hours. The cream was removed from the top. These steps are repeated three more times to obtain skim milk. Fresh overnight culture of bacteria was inoculated into 10%skim milk and incubated at 37°C for 48 hours.[2]
7) *Lactose Utilisation Test:* Media was prepared using 1gram of peptone, 1.5gram of NaCl, 0.0018gram of phenol red, 0.5gram of lactose in 100ml distilled water and the pH of the media is 7. The organism was inoculated into the media and incubated at 37°C for 48 hours in rotary incubator.[2]
8) *Antibiotic Sensitivity Test:* The Antibiotic Sensitivity test was performed by Disk diffusion method. The isolated sample of bacteria was swabbed in MRS Agar plate. 30µg concentration Erythromycin, Gentamycin and Amoxicillin were used for this experiment.[1]
9) *Antibacterial Activity:* Antimicrobial activity of isolated bacteria was observed by agar well diffusion method. Fresh overnight broth culture of the pathogen Escherichia coli, Staphylococcus aureus Salmonella spp. Klebsiella spp. were swabbed onto different MRS agar plates and wells were made in each plate and the wells were filled with 100µl and 50µl of isolated Lactobacillus culture respectively. The plates were incubated at 37°C for 24 hours. The zone of inhibition was measured.[2]
10) *Lyophilization of The Probiotic Bacteria:* The organism was inoculated in 2000ml of MRS broth. It was then freeze dried and made to powdery form using lyophilization technique.[2]
11) *Preparation of Milk and Dark Chocolate:* For the preparation of milk chocolate double boiler method is used, heat 105 gram of cocoa butter till it melts. After it gets melted add 200 gram of milk powder, 85 gram of honey and 118 gram of cocoa powder and mixed well. Then 1 teaspoon of Vanilla extracted is added and mixed well. It is then allowed to cool. For the preparation of dark chocolate by double boiling method 105 gram of cocoa butter is heated and melted. Then 118gram of cocoa powder and 85 gram of honey is added and mixed well. It is then added with 1 teaspoon of vanilla extract and mixed well and allowed to cool.[2]
12) *Application of Probiotic Bacteria in Foods:* When both varieties of chocolate after preparation reaches the normal room temperature the powdery form of Lactobacillus sp., is added mixed well and immediately stored in freezer.[2]

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III. RESULTS AND DISCUSSION

1) *Identification of the Isolated Organism*



(Figure.1)

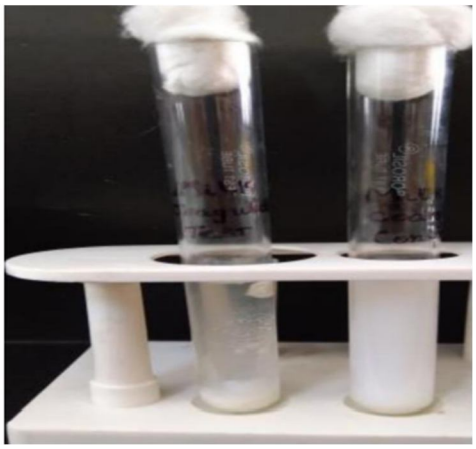
2) *Gram Staining:* The bacteria was gram positive, rod shaped occurring singly or in chains. The gram staining results indicated that the bacteria could be identified as Lactobacillus spp. [2]
3) *Endospore Staining:* The isolated bacterium was non spore forming.[2]
4) *Motility Test:* (Hanging drop method) The isolated bacteria were non motile which is characteristic of Lactobacillus acidophilus[2].
5) *Biochemical Characteristics:* Biochemical characteristics of the bacterial strain were determined by using various biochemical tests like catalase, oxidase, Indole test, Methyl Red test, Voges-Proskauer test, Citrate utilization test, Urease test, starch hydrolysis test etc., and their results were recorded in table 1.[2]

Sr.No	BIOCHEMICAL TEST RESULT
1	Catalase test- Negative
2	Oxidase test - Negative
3	Indole test - Negative
4	Methyl Red test - Negative
5	Voges-Proskauer - Negative
6	Citrate utilization test - Negative
7	Urease test - Negative
8	Starch hydrolysis test- No zone of hydrolysis

6) *Carbohydrate Fermentation Test:* The isolated Lactobacillus spp., were able to ferment all the tested sugars. The colour of the medium changes from red to yellow.[2]

1	Glucose - Positive
2	Lactose- Positive
3	Sucrose – Positive
4	Mannitol - Positive

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(Figure.2)
Carbohydrate Fermentation Test

7) *NaCl Tolerance Test:* The organism was able to tolerate NaCl. The turbidity of the culture was measured at 620nm.[2]
NaCl tolerance test:- NaCl TOLERANCE TEST OD
Value :- 2.222.

8) *Phenol Tolerance Test:* The organism was able to tolerate phenol and grow. The OD value of the culture measured at 620nm.[2]
Phenol tolerance test: - PHENOL TOLERANCE TEST OD
Value: - 2.105.

9) *Milk Coagulation Assay:* Coagulation of milk was observed in the tube inoculated with the test organism.



(Figure.3)
Milk Coagulation Assay

10) *Lactose Utilization Test:* Lactose utilization test is positive for Lactobacillus as the color is changed from yellow to red.[2]
11) *Antibiotic Sensitivity Test:* The organism was sensitive to all the three selected antibiotics (table 5). Table 5- Antibiotic sensitivity test.[2]

Sr.No: Antibiotics Sensitivity Test

1 . Erythromycin 23mm
2 . Gentamycin 17mm
3 . Amoxicillin 11mm



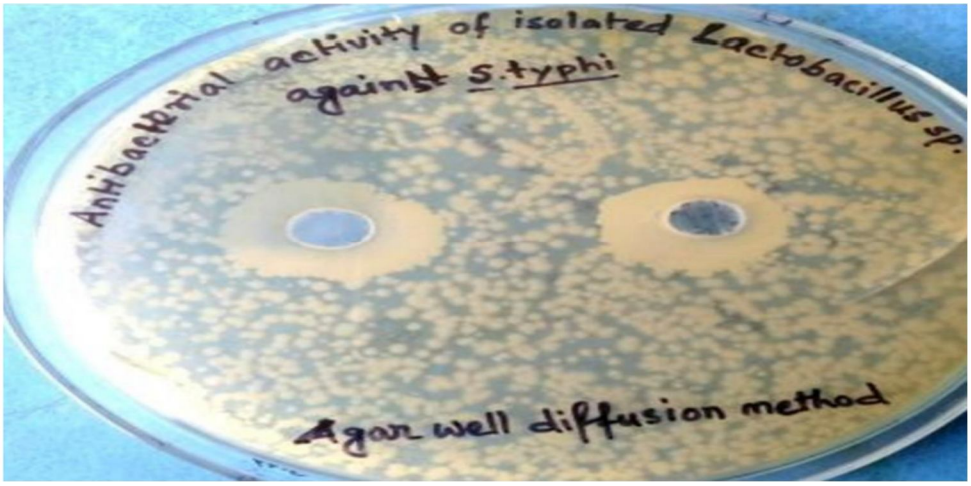
(Figure.4)
Antibiotic Sensitivity Test

A. Detection of Antibacterial Activity

Antibacterial activities exhibited by Lactobacillus species which indicates that the cell free solution of isolated Lactobacillus species were able to inhibit the growth of all the test microorganisms. This experiment clearly indicates that the inhibitory metabolites produced by isolated . Lactobacillus species were extracellular and diffusible. These results are shown in the . The experimental results showed that the traditional fermented milk product curd contain Lactobacilli which can tolerate inhibitory substances and were able to survive both in acidic and alkaline conditions. They exhibited antibacterial activity against some indicator pathogens. Based on these characteristics the isolates may have potential for natural preservatives and may also be considered for probiotic application. Table 4 Antibacterial activity of isolated Lactobacillus spp. by agar well diffusion method.[1]

B. Test Organisms Zone of inhibition (mm)

Salmonella typhi(MTCC 890) 19
Escherichia coli (MTCC 443) 17
Staphylococcus aureus (MTCC 3160) 14
Klebsiella pneumonia(MTCC 109) 17.[1]



(Figure.5)
Antibacterial Activity

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C. Lyophilization and Preparation of Probiotic Chocolate

By using lyophilization technique the Lactobacillus spp. were freeze dried. The freeze-dried Lactobacillus spp. were in powdery form. This powder was added at thelast step of preparation of chocolate. The probiotic chocolate is prepared. The chocolate is easily assimilated and it is better to eat a chocolate than medicinal tablets. The lyophilized freeze dried of Lactobacillus spp. the powder addition to the chocolate preparation andafter preparation of chocolate.[1]



(Figure.6)

a) The lyophilized freeze dried of Lactobacillus spp. b) Powder addition to the chocolate preparation c) Preparation of probiotic chocolate d) After packaging of probiotic chocolate.[1]

IV. SUMMARY AND CONCLUSION

The proximate analysis of both sweet potato and pineapple peels shows that the fermented peels have high nutritional content of protein, moisture, ash and fat while carbohydrate and crude fibre content reduced after fermentation for both substrate this shows that fermentation has a great influence and can bring a desirable change into product.[11]

The purpose of this study was to isolate and identify comparatively most promising probiotic strains for milk fermentation that can facilitate both qualitatively and quantity timely the manufacturing of functional foods. Fifteen Lactobacillus strains were initially isolated and only four strains were examined for morphological and biochemical characterizations due to their ability of gas production in Durham tubes. This study demonstrated that two Lactobacillus strains isolated from buffalo milk showed the best probiotic potential.[12]

Probiotics are widely used in order to solve and simplify particular diseases. The net benefit of probiotics during the perinatal period in preventing allergic disease has lead to a World Allergy Organization recommendation on probiotic use during pregnancy, breastfeeding, and weaning in families with a high risk of allergic disease. Future research must investigate the mechanisms by which gut microflora interacts with the intestinal epithelium in health and disease. With this knowledge, optimal probiotic strains can be developed. The viability of probiotics is a key parameter for developing probiotic food products. New technologies have been developed to enable high cell yield at large scale and ensure probiotic stability for a long period in food.[13]

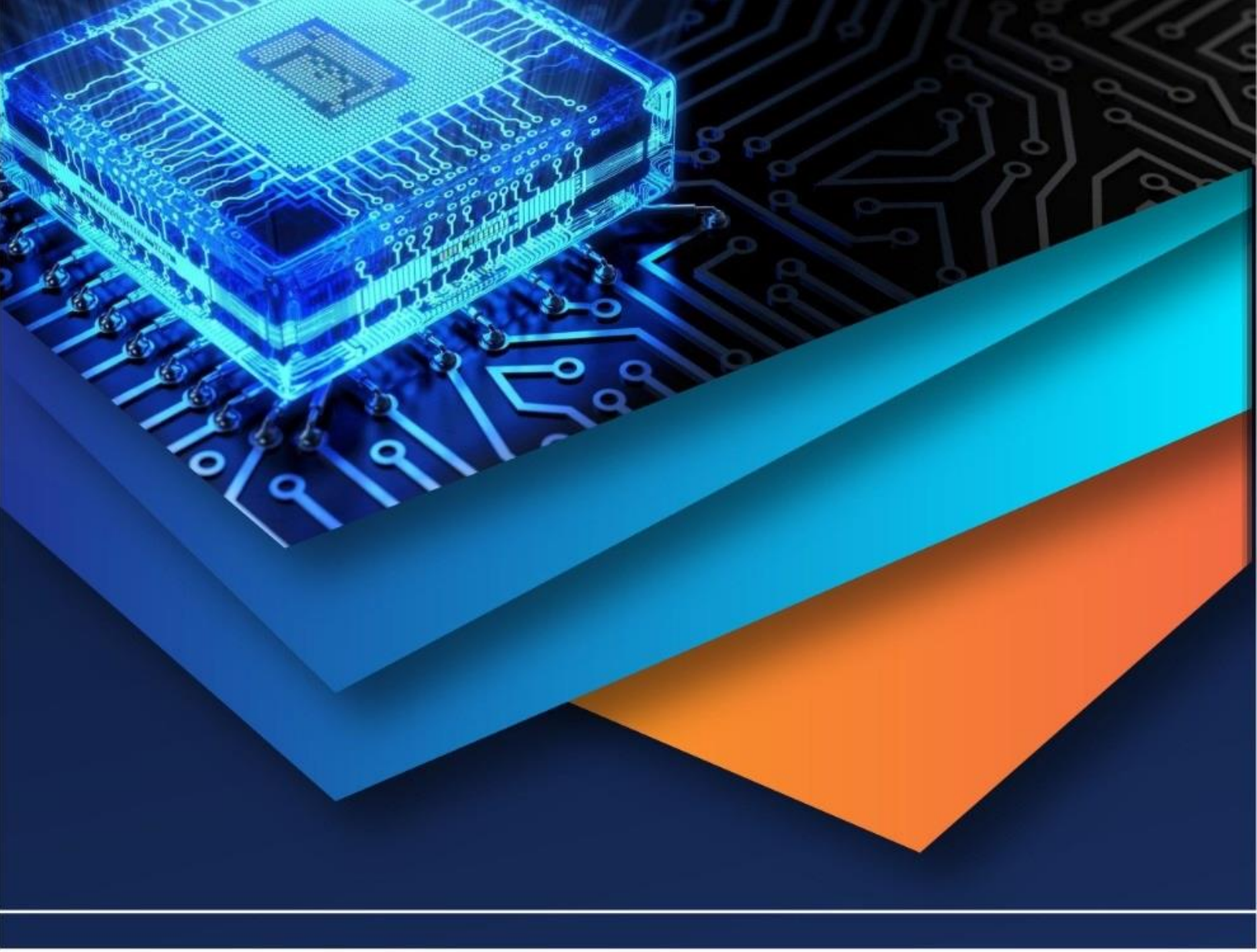
A. Clinical Applications of Probiotics on Human Health

1) *Colorectal Cancer Prevention:* Although diet is thought to contribute to the onset of colorectal cancer, and both probiotics and prebiotics have been shown to improve biomarkers associated with colorectal cancer, there are limited data in humans showing any benefit of probiotics or prebiotics in the prevention of colorectal cancer.[13]
2) *Probiotics and Allergy:* Allergies are misguided reactions of the immune system in response to (what should be harmless) particles. Probiotics treat allergies by healing your damaged digestive system, which decreases inflammation, stabilizes your immune system, and strengthens your gut lining. An allergy is a hypersensitivity reaction initiated by immunological mechanisms. Probiotics modify the structure of antigens, reduce their immunogenicity, intestinal permeability and the generation of proinflammatory cytokines that are eminent in patients with a diversity of allergic disorders.[13]

3) *Probiotics and Blood Pressure:* It has also been demonstrated that probiotics and their products can improve Blood pressure through mechanisms including improving total cholesterol and low-density lipoprotein cholesterol levels Reducing blood glucose level and insulin resistance, regulating the renin–angiotensin system and significant reduction takes place in blood or serum cholesterol when cholesterol is elevated Probiotics and Voice Prosthesis Probiotics emphatically diminish the occurrence of pathogenic bacteria in voice prosthetic biofilms. Effectively disposes of biofilm development on indwelling voice prostheses, possibly related to the presence of Streptococcus thermophiles and Lactobacillus.[13]
4) *Immunologic Enhancement/ Immunity Stimulation:* Probiotics have biological effect in Immunological functionality. The immunological benefits of probiotics can be due to activation of local macrophages and modulation of IgA production locally and systemically, to changes in pro/anti-inflammatory cytokine profiles, or to the modulation of response towards food antigens The intrinsic properties of lactobacilli to modulate the immune system make them appealing for wellbeing applications.[13]
5) *Probiotics and HIV:* Probiotics appear to support maintenance of a strong gut epithelia layer, improve gut barrier function and stimulation of innate immunity which act as the first layer of defense against translocation of viral particles and bacterial pathogens. When immune system is well developed, able to prevent HIV replication and slow down the progression of AIDS in host. Daily consumption of probiotics over a prolonged period of time can improve CD4 count in people living with HIV. A screening of saliva taken from several volunteers demonstrated that some Lactobacillus strains created proteins that are fit for binding a specific type of sugar, called mannose, found on HIV envelope. The binding of the sugar empowers the microscopic organisms (bacteria) to adhere to the mucosal coating of the mouth and gastric tract and colonize them. One of the strains indicated copious mannose-binding protein particles into its surroundings which binded to the sugar coating henceforth neutralizing HIV.[13]

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