



ISOLATION AND CHARACTERIZATION OF LACTIC ACID BACTERIA FROM DONKEY'S MILK

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Abstract

Probiotics are live microorganisms promoted with claims that they provide health benefits when consumed, generally by improving or restoring the gut microbiota. Probiotics are considered generally safe to consume. Lactic acid bacteria (LAB) represent a group of micro-organisms that are naturally present in many foods and in the gastrointestinal and urogenital tract of animals. It has been shown that these LAB can produce Antimicrobial compounds. Milk samples from different donkeys were collected and the samples were serially diluted. Pour plate technique was done on MRS agar plate and incubated at 37°C for 24 hrs. The isolated bacteria was used to produce consumables that develops immunity in the body. It contains essential amino acids, fatty acids and taurine.

Keywords: Donkey milk , Lactic Acid Bacteria , 16S rRNA Sequencing , Nutritional value , Gut microbiota

1.Introduction

Donkey milk is the milk from the domesticated donkey. It has been used since antiquity for cosmetic purposes as well as infant nutrition. Donkey milk has been used by humans for alimentary and cosmetic purposes since Egyptian antiquity. Doctors recommended it to treat several afflictions, due to its healing and cosmetic virtues.

Donkey milk (DM) has gained scientific attention in recent years. Because it has compelling nutrients and functional elements. (Mansueto *et al.* 2013 There are much similarities in chemical compositions of human milk and donkey milk. Donkey's milk is used as an alternative for infants with multiple food allergies and

intolerances. Recently, fermented beverages based on donkey milk were proposed as important sources of probiotics and antioxidants with several health benefits.

Donkey milk contains high levels of lactose and minimal amounts of casein and fat. One of the main particularities of Donkey milk is its high lysozyme's content. Lysozyme shows antibacterial activity against numerous Gram-positive bacteria; including lactic acid bacteria (LAB).

Recently, overuse of antibiotics has led to the emergence and spread of high bacterial resistance, making difficult to treat infections involving tremendous research efforts by the medical and scientific community (Chiavari *et al.* 2005). In fact, living organisms have developed an immense molecular diversity, containing ubiquitous low molecular weight secondary metabolites isolated from plants, insects, marine organisms, and other microorganisms, including lactic acid bacteria (LAB). LAB are microorganisms forming a group composed of bacilli and cocci. The common characteristic of these bacteria is the production of lactic acid, which is the end product of the fermentation process of several sugars.

The germs represent a potential source of several metabolites with antimicrobial and anti fungal activities like organic acids, reuterin, hydrogen peroxide, diacetyl carbon dioxide, and bacteriocins. They are present in different microbial biotopes such as soil, plants, the digestive system of humans, and dairy products (Chiavari *et al.* 2005).

Donkey milk is largely used in the manufacture of soaps and moisturisers, but new evidence show its possible medical use, especially to treat, under the supervision of a doctor, infants and children with cow's milk protein allergy (CMPA) and with appropriate precautions such as a natural "formula" for infant.

The whey protein of donkey milk is high. Moreover Donkey milk has a lot of unsaturated fatty acids, especially linoleic acid, and low content of fat and cholesterol. It is rich in calcium and selenium (Cavallarin *et al.* 2015.) Donkey milk also has strong antioxidant activity, retarding the ageing process, and is rich in kinds of immune boosting substances to the human immune system. The medicinal value of donkey milk was recorded on both valuable prescriptions for Emergencies by Sun Simiao in the Tang Dynasty and Compendium of Materia Medica by Li Shizhen in the Ming Dynasty. In Peru, donkey milk is used to treat diseases like asthma, bronchitis, diabetes, anabrosis, gastritis, and even ease climacteric discomfort. There are also many people treating tuberculosis and gastric ulcer by drinking donkey milk in Xinjiang, China. In some countries in Europe and America, donkey milk is not only the component of many biological products, but also a kind of health care products, accepted by more and

more people. To summarise, donkey milk has high nutritional value and wide medicinal value.(Andrighetto *et al.* (2002))

As an insoluble protein, casein is hard to be digested and can form larger and harder clots in the baby's stomach. However whey protein, belonging to soluble protein, only forms small and soft clots in an infant's stomach, being easy to digest and absorbed. Furthermore whey protein contains a variety of biological activity proteins, enzymes, peptides, immune factors and growth factors, which plays an important role in growth and mental development of humans. The nutritional value and biological potency of whey protein are very high, therefore it is praised as “the king of protein”. The proportion of whey protein and casein in milk relates to the nutrition and absorption for the body. In the body, lactose can be converted to glucose which provides energy and participates in the composition of tissues and organs, and galactose which can synthesise glycolipid, one of basic materials for neural tissue, and has a vital role in the development of infant nervous systems. In addition, lactose can promote the proliferation of intestinal probiotics, inhibit the growth of spoilage bacteria, improve the absorption of calcium, phosphorus and other mineral elements, and boost the quality and strength of the skeleton.

The yield of donkey milk is relatively low. A donkey only produces 100–150 kg in one lactation period for processing and usage. But donkey milk has special value in scientific research and breeding efficiency. In China, donkeys are mainly located in the northwest region whose geographical location is relatively remote and economical development is relatively lagging(Ruaro *et al.* 2013). Because of the difficulties of milk acquisition, preservation, transportation and ethnic customs, donkey milk's development has not been taken seriously. The nutritional values of donkey milk have not been well studied, and systemic research is still lacking. Nowadays, there is no acceptance criteria of donkey milk, therefore appropriate standards are required to be set for standardising the donkey milk market, strengthening the donkey milk product quality inspection and market regulation, and maintaining rights of consumers and businesses.

Therefore, the development and utilisation of donkey milk has great economic value and social value. However the nutrients and functions of donkey milk are not well studied and basic data need to be collected, which will restrict the development of the donkey milk industry. Thus, the donkey milk powder was obtained in the study. The composition of conventional nutrients, amino acids, fatty acids, and antioxidant activity of donkey milk and powder were studied. It would shed light on the development of the donkey milk industry, improvement of the comprehensive utilisation ratio of donkey milk resources, arousing the enthusiasm of raising donkey, increasing the income of farmers and herdsmen, and the promotion of economic development.(Perna *et al.* (2015))

4. MATERIALS AND METHODS

4.1. Sample collection and isolation of lactic acid bacteria

Seven samples of raw donkey milks were collected from lactating donkeys in the surrounding area of Tirupur & Erode district. Samples were collected using sterile bottles and stored in an icebox until delivery of the laboratory for analysis.

368580952.368579608. Isolation of Lactic acid bacteria

About 1 ml of milk sample was mixed with 9 ml of saline [8.5g / L] to make an initial dilution [10⁻¹]. The suspension was used for making suitable serial dilutions up to 10⁻⁸. Enumeration of LAB was determined using MRS (Man de Rogosa Sharpe) agar and M17 agar medium by pour plate [1 ml in 15 ml medium] incubated at 37 ° C for 24 hours. After incubation colonies were chosen based on their morphology on MRS agar plate.

368580112.368580616. Morphological and general characterisation of LAB:

Simple tests such as gram staining, catalase test, sugar fermentation test were performed for isolates.

368580112.368580616.368580168. Gram staining(Singh *et al.*, 2014)

The isolates are grown in freshly prepared media and incubated overnight. After incubation the cells were taken and then gram staining procedure was followed. The gram reaction of the isolates was determined by light microscopy.

LAB found till now have been known to be gram positive and it shows blue-purple colour after staining.

368581960.368581568.368582016. Catalase and oxidase test (Singh *et al.*, 2014)

Catalase enzyme produced by many microorganisms that breaks down the H₂O₂ into water and oxygen that releases O₂ gas bubbles. The formation of gas bubbles indicates the presence of catalase enzymes.



Cultures were grown overnight on MRS broth. The fresh liquid cultures were also used for catalase activity by dropping 3% hydrogen peroxide solution onto 1 ml of overnight cultures and their catalase activity was observed.

368582800.368581680. Physiological and biochemical characterisation of Lactic acid bacteria: 4.4.1.Sugar fermentation(Thakkar *et al.*, 2015)

MRS broth supplemented with different Sugars (glucose, lactose and maltose) and phenol red as pH

indicator was inoculated with active cultures at 1%, incubated at 37°C for 24 hours. The cultures were identified based on acid and gas production after the incubation period.

1. **Growth at different pH**(Thakkar *et al.*, 2015)

To check the growth of isolates at various pH, MRS broth supplemented with different pH 2.0, 3.0, 7.5, 8.0 was prepared, 1% of fresh culture was inoculated and then incubated at 37°C for 28 hours. During incubation, extent of growth was recorded objectively based on visible turbidity marked as double positive sign (++) for maximum growth, single positive sign (+) for normal growth and negative sign (-) for no growth.

1. **Growth at different temperatures** (Thakkar *et al.*, 2015)

Overnight grown active cultures were inoculated at 1% in MRS broth tubes and incubated up to 7 days at 15, 37, 45 and 55°C. Extent of growth was visually recorded based on intensity of turbidity.

2. **Growth at different NaCl concentration** (Thakkar *et al.*, 2015)

Overnight grown active cultures were inoculated at 1% in MRS broth tubes adjusted to various concentrations of NaCl viz. 3.5, 6.5 and 18% (w/v) along with their respective controls. The cultures were incubated at 37°C. After 24 hours of incubation, extent of growth was recorded objectively based on visible turbidity marked as double positive sign (++) for maximum growth, single positive sign (+) for normal growth and negative sign (-) for no growth.

1. **Probiotic Strain Selection Test**

1.1. **Blood Haemolysis test**(Mohmad *et al.*, 2015)

As the strains were isolated from food material, blood haemolysis test was performed, to eradicate any chance that our isolates may be pathogenic. It is also one of the criteria for assessing the safety of use of probiotics as food supplements. Pathogens produce highly toxic substances which lyse the RBC and form a clear zone around them.

The haemolytic activities of isolated strains were determined according to as follows: all examined strains were separately grown in MRS broth at 37°C for 24 hours and then streaked onto Columbia agar base plates supplemented with 5% (v/v) whole human blood. The plates were incubated aerobically at 37°C for 48 hours. then observed the clear zones and the colour of haemolysis around the growth colonies. All experiments were performed in three replicates.

368582968.368582912. **Antibiotic susceptibility test**(Singh *et al.*, 2014)

Probiotic strains must be sensitive towards antibiotics. There is a light risk that antibiotic resistance probiotic strain may transfer the antibiotic resistance genes to the pathogens via transformation in the gut. Due to any chance resistant pathogens get introduced into the human via the food chain and cause serious

problems. Sensitivity of probiotics strains towards the antibiotics being tested by using Kirby - Bauer disc diffusion technique. Used antibiotics for testing: Tetracycline, Penicillin, Vancomycin, Streptomycin and Kanamycin. For this purpose MRS agar inoculated with LAB and disc were placed. After the incubation period (24 hours/ 37°C) and inhibition zones were observed to determine the antibiotic resistance of isolates.

368582968.368582913. **Antagonistic activity**(Bolanle *et al.*, 2015)

The agar overlay method was employed to determine the ability of the viable lactic acid bacteria strains to inhibit the growth of the indicator pathogens, *E.coli* and *Staphylococcus aureus*.

A loop full of LAB in MRS broth was inoculated on MRS agar plate as a thick line of about 2 mm and about 30mm long at a good away from the edge of the plates and incubated under micro aerophilic condition at 37°C for 24 hours. After incubation, the MRS agar plates were overlaid with approximately $0.2\text{ml} \times 10^{-7}$ CFU /ml of an overnight broth culture of the test pathogens inoculated in 10 ml of Muller Hinton soft agar (with 0.7% agar-agar). The overlay was allowed to set and incubated at 37°C under aerobic condition. The plates were then examined for zones of inhibition around the line of the LAB and the clear zones were measured.

1. Probiotic Viability in Gastrointestinal tract:

1.1. Tolerance to Bile Salts (Hawaz.E. 2014)

The method described by Mabrouk et al was used to determine the tolerance of examined lactic acid bacteria to bile salts. MRS medium containing 0.1 & 0.3% (w/v) bile concentration was inoculated with overnight culture of lactic acid bacteria. Viable colonies were counted for 1, 2, 24 hours incubation time on MRS agar and also growth was monitored at OD 620 nm.

1.2. Phenol resistance tests (Thakkar *et al.*, 2015)

Overnight grown active cultures were inoculated at 1% in MRS broth tubes adjusted to various concentrations of phenol level viz. 0.4, 0.5 and 0.6 % (w/v) along with the respective controls. The cultures were incubated at 37°C. After 24 hours of incubation, extent of growth was recorded objectively based on visible turbidity marked as double positive sign (++) for maximum growth, single positive sign (+) for normal growth and negative sign (-) for no growth.

368584816. Anti microbial activity

The agar overlay method was employed to determine the ability of the viable lactic acid bacteria strains to inhibit the growth of the indicator pathogens, *E.coli*, *Staphylococcus aureus* and *Candida spp.*, A loopful of LAB in MRS broth were inoculated on MRS agar plate as a thick line of about 2 mm and above 30 mm long at a good distance away from the edge of the plates and incubated under micro aerophilic conditions at 37°C for 24 hours. After incubation, the MRS agar plates were overlaid with approximately 0.2 ml x 10^7 CFU/ ml of an overnight broth culture of the test pathogens inoculated in 10 ml of the Mueller Hinton

Agar (with 0.7% agar-agar). The overlay was allowed to set, and incubated at 37°C under aerobic conditions. The plates were then examined for a clear zone of inhibition around the line of LAB and the clear zones were measured.(Adeniyi *et al.*,2015)

368583248. Bacteriocin activity

The selected strains were grown in MRS broth at 37°C for 24 hrs. Cell free supernatants were collected by centrifugation (8500 rpm, 10 min, 4°C) of overnight MRS broth cultures.. The same supernatants without the pH adjustment were used for the bacteriocin activity. The MRS broth with pH 7 and MRS broth without pH adjustment were used as the control and all culture supernatants were filter sterilised to eliminate the possible presence of viable cells (modified).The inhibition activity was examined by means of the diameters of inhibition zones using the agar well diffusion method. Briefly 10, 15, 20 µl of the cell free supernatants were placed into wells on the appropriate media agar plates which are seeded with indicator strains, *E. coli*, *Staphylococcus aureus*. After incubation of 24 hrs the diameters of the inhibitory zones were measured.(Musikasang *et al.*,2012)

368584817. Column Chromatography

The isolates were grown in a production media, MRS media for 24 hrs at 37°C. Then it was centrifuged at 8000 rpm for 10 minutes. 400g of ammonium sulphate per litre of culture supernatant was added and allowed to settle for 24 hrs at 4°C. The protein precipitate was collected by centrifugation at 6000 rpm / minute for 20 min and dissolved in 50 ml of 20 mM/L sodium phosphate buffer (Ph 6.0).

Further it was applied on Diethylaminoethyl-cellulose column (1.5 x 40.0 cm) equilibrated with 0.1 M/L Tris – HCL buffer (pH 9) and eluted with linear salt gradient of NaCl (0-1 mol/l). The Active fractions were pooled together, concentrated by ammonium sulphate, loaded on Sephadex G-75 column (1.5940 cm) equilibrated with 0.1 mol/L Tris-Hcl buffer (pH 9) and eluted with the same buffer at a flow rate of 0.5 ml/min.(Elayaraja *et al.*, 2014)

368583864. SDS- PAGE

An intact SDS PAGE system should include: a tank, lid with power cables, electrode assembly, cell buffer dam, casting stands, casting frames, combs (usually 10-well or 15-well), and glass plates (thickness 0.75mm or 1.0mm or 1.5mm). (Bio-rad brand one is recommended).

The SDS PAGE gel in a single electrophoresis run can be divided into stacking gel and separating gel. Stacking gel (acrylamide 5%) is poured on top of the separating gel (after solidification) and a gel comb is inserted in the stacking gel. The acrylamide percentage in SDS PAGE gel depends on the size of the target protein in the sample.

368584032. **Results and discussion**

368584032.368585040. **Isolation of Lactic acid bacteria:**

About 1 ml of milk sample was mixed with 9 ml of saline [8.5g / L] to make an initial dilution [10⁻¹]. The suspension was used for making suitable serial dilutions up to 10⁻⁸. Enumeration of LAB was determined using MRS (Man de Rogosa Sharpe) agar and M17 agar medium by pour plate [1 ml in 15 ml medium] incubated at 37 ° C for 24 hours. After incubation colonies were chosen based on their morphology on MRS agar plate.

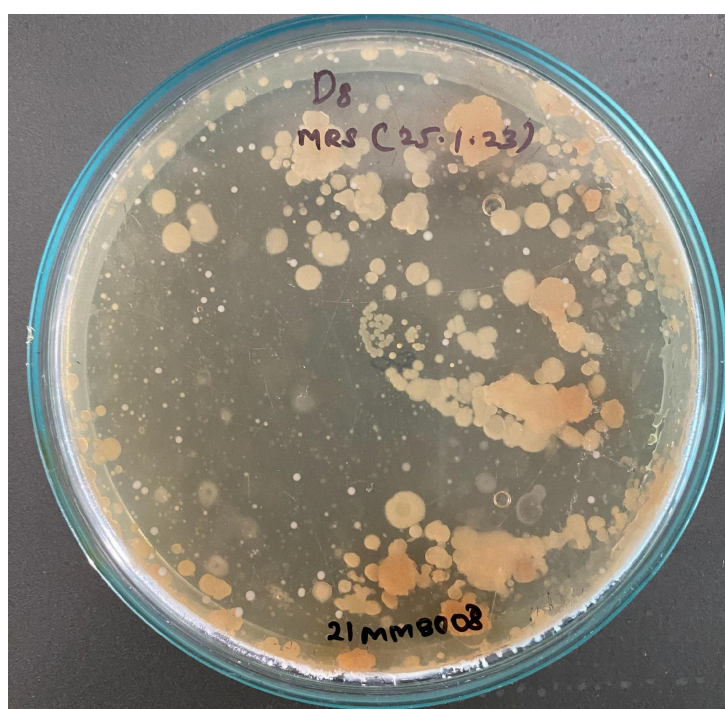


Figure 1.1 The plate shows the presence of bacterial colonies on MRS agar plates.

368584032.368585041. **Gram staining**

The isolates are grown in freshly prepared media and incubated overnight. After incubation the cells were taken and then gram staining procedure was followed. The gram reaction of the isolates was determined by light microscopy. LAB found till now have been known to be gram positive and it shows blue-purple colour after staining.

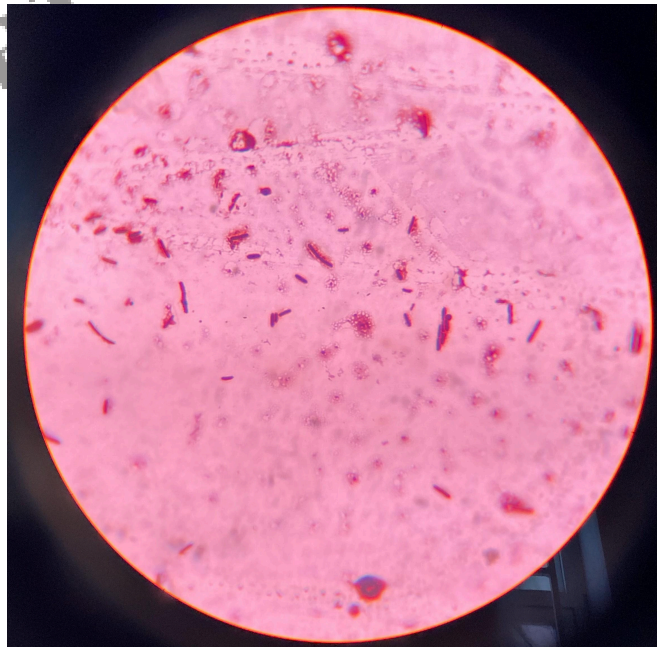


Figure 1.2 The image shows the presence of **gram positive rods**.

368585264.368584984. Catalase and oxidase test

Catalase enzyme produced by many microorganisms that breaks down the H₂O₂ into water and oxygen that releases O₂ gas bubbles. The formation of gas bubbles indicates the presence of catalase enzymes.

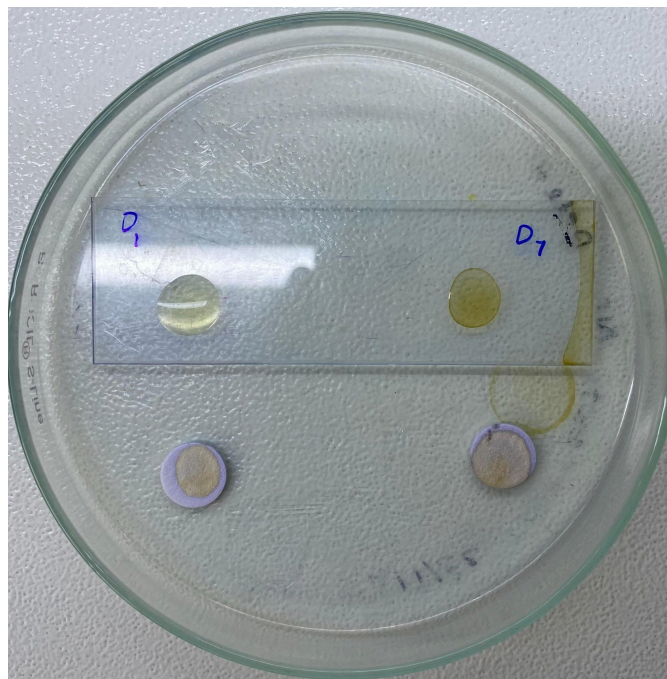


Figure 1.3 The image shows **negative** results for catalase and oxidase tests.



Cultures were grown overnight on MRS broth. The fresh liquid cultures were also used for catalase activity by dropping 3% hydrogen peroxide solution onto 1 ml of overnight cultures and their catalase activity was observed. No gas bubbles were observed and hence the sample shows negative results for catalase test.

316368944.316368832. Sugar fermentation

MRS broth supplemented with different Sugars (glucose, lactose and maltose) and phenol red as pH indicator was inoculated with active cultures at 1%, incubated at 37°C for 24 hours. The cultures were identified with acid and gas production after the incubation period.

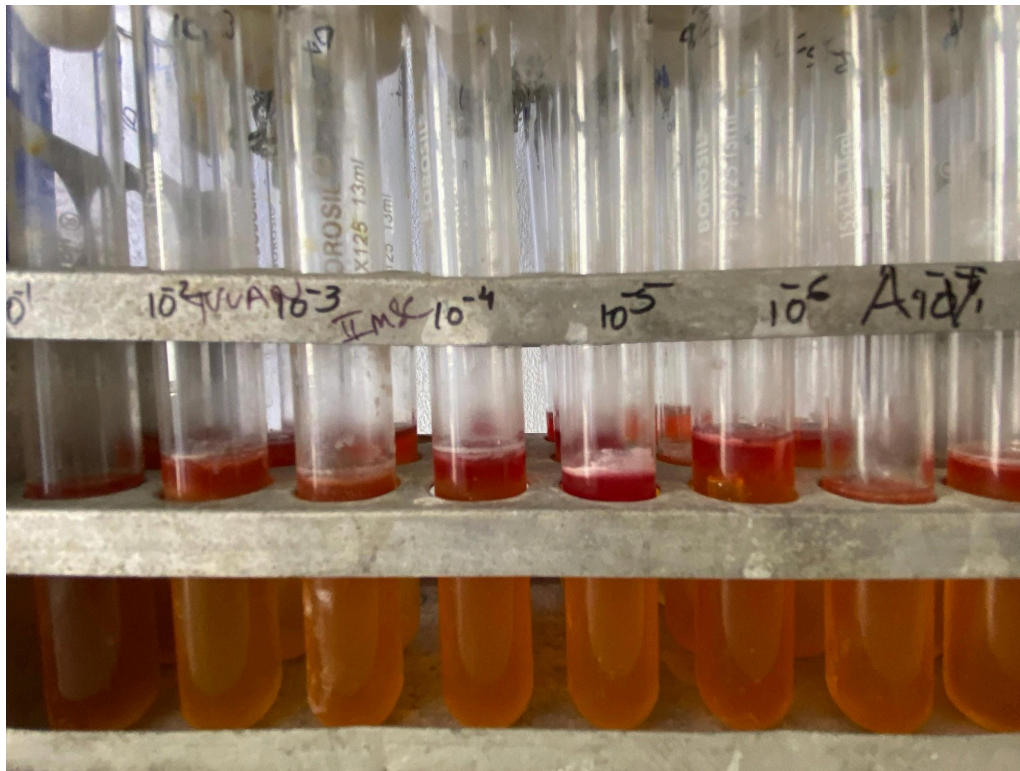


Figure 1.4 The image shows the **production of acid and gas** in Durham's tube

316368496.316367880. Blood Haemolysis test

As the strains were isolated from food material, blood haemolysis test was performed, to eradicate any chance that our isolates may be pathogenic. It is also one of the criteria for assessing the safety of use of probiotics as food supplements. Pathogens produce highly toxic substances which lyse the RBC and form a clear zone around them.

The haemolytic activities of isolated strains were determined according to (Marakoudakis et al., 2009) as follows: all examined strains were separately grown in MRS broth at 37°C for 24 hours and then streaked onto Columbia agar base plates supplemented with 5 % (v/v) whole human blood. The plates were incubated aerobically at 37°C for 48 hours. then observed the clear zones and the colour of haemolysis around the growth colonies.



Figure 1.5 No haemolysis were observed in blood agar plates

316368328.316368160. Antibiotic susceptibility test

Probiotic strains must be sensitive towards antibiotics. There is a light risk that antibiotic resistance probiotic strain may transfer the antibiotic resistance genes to the pathogens via transformation in the gut. Due to any chance resistant pathogens get introduced into the human via the food chain and cause serious problems. Sensitivity of probiotics strains towards the antibiotics being tested by using Kirby - Bauer disc diffusion technique. Used antibiotics for testing: Tetracycline, Penicillin, Vancomycin, Streptomycin and Kanamycin. For this purpose MRS agar inoculated with LAB and disc were placed. After the incubation period (24 hours/ 37°C) and inhibition zones were observed to determine the antibiotic resistance of isolates.

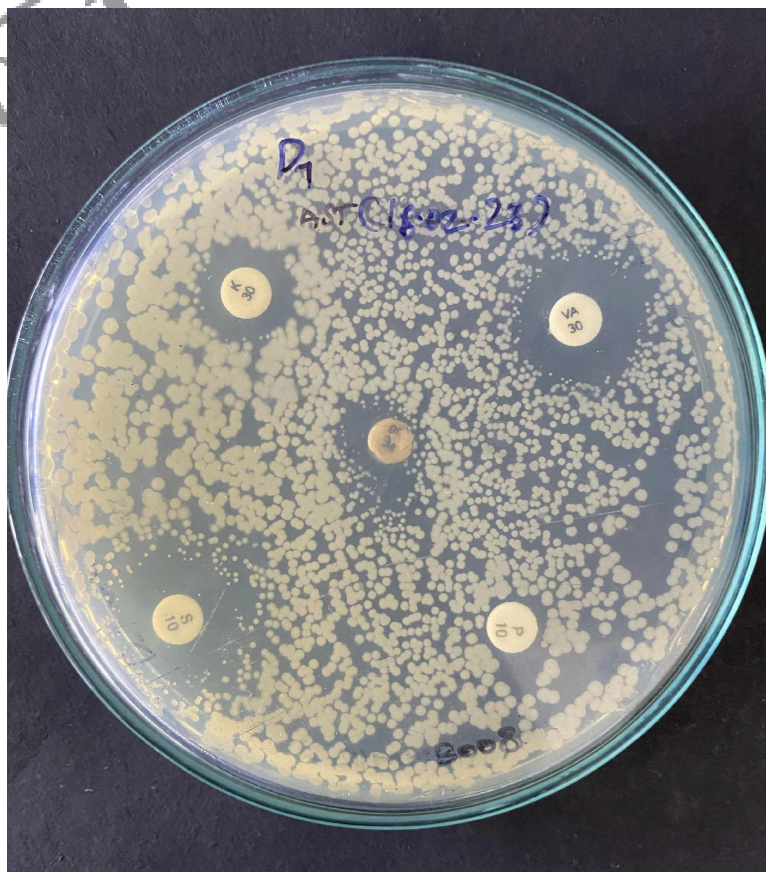


Figure 1.6 Zones of inhibition were observed on MHA

Antibiotic/ Sample	D o n k e y 1 (C m)	D o n k e y 2 (C m)	D o n k e y 4 (C m)	D o n k e y 7 (C m)
Penicillin	0	0	1	0.8
Streptomycin	1	2.1	2	2
Kanamycin	1	0	1.8	1.2
Vancomycin	1.2	1	0	1.8
Tetracycline	1.8	1.7	2.5	1.1

316368104.316368216. Phenol resistance test

Overnight grown active cultures were inoculated at 1% in MRS broth tubes adjusted to various concentrations of phenol level viz. 0.4, 0.5 and 0.6 % (w/v) along with the respective controls. The cultures were incubated at 37°C. After 24 hours of incubation, extent of growth was recorded objectively based on visible turbidity marked as double positive sign (++) for maximum growth, single positive sign (+) for normal growth and negative sign (-) for no growth.

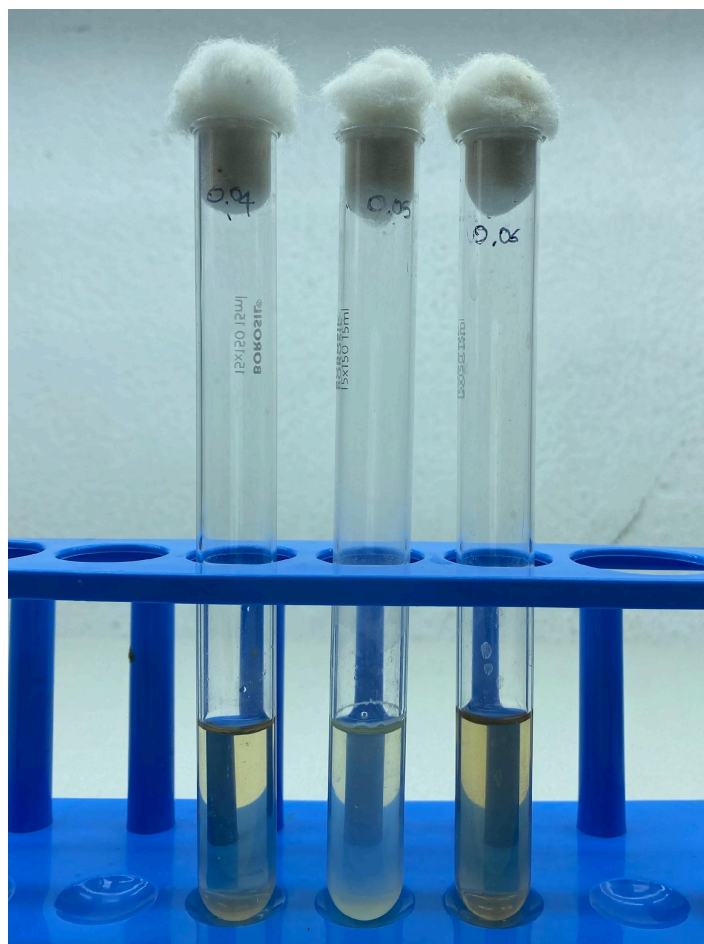


Figure 1.7 Turbidity was **observed in 0.5** concentration of phenol

S a m p l e / D i l u t i o n	0.4	0.5	0.6
D1	-	-	-
D2	-	++	-
D4	-	-	-
D7	-	++	-

316369616.316369728. Growth at different NaCl concentration

Overnight grown active cultures were inoculated at 1% in MRS broth tubes adjusted to various concentrations of NaCl viz. 3.5, 6.5 and 18% (w/v) along with their respective controls. The cultures were incubated at 37°C. After 24 hours of incubation, extent of growth was recorded objectively based on visible turbidity marked as double positive sign (++) for maximum growth, single positive sign (+) for normal growth and negative sign (-) for no growth.

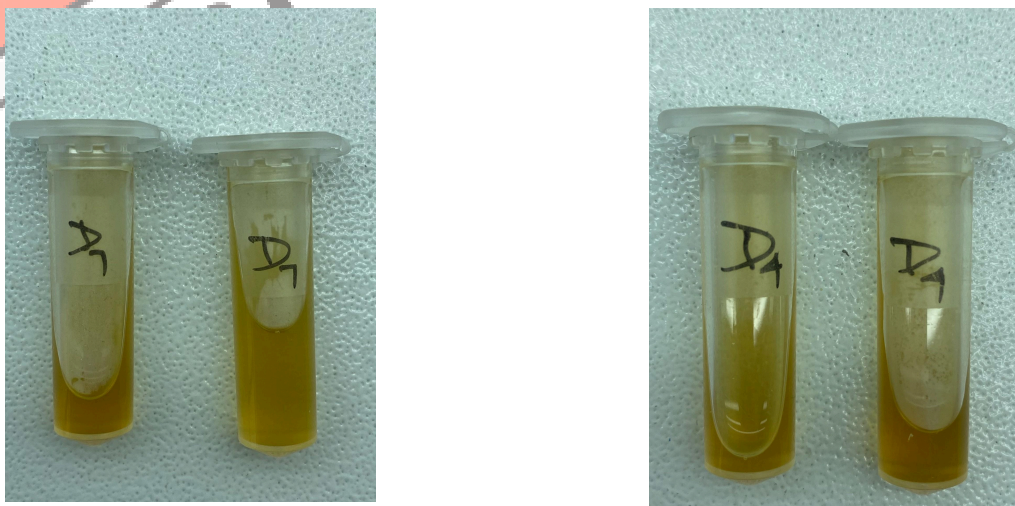


Figure 1.8 The image shows the samples with and without NaCl

Sequencing report

16S rRNA sequencing

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GGGGGGTGCTAATACATGCAAGTCGAACGCTTTTCTTTCACCGGAGCTTGCTCCACCG
AAAGAAAAGAGGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCATCAGAAGG
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The organism isolated is found to be : Enterococcus gallinarum