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(RESEARCH ARTICLE)

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Molecular identification of bacteria (*Klebsiella aerogenes,* PP335235) isolated from street food (Fried Rice); study of its antibiotic sensitivity pattern and probiotic fortification of fruit juice (*Fragaria ananassa, Passiflora ligularis* and *Phyllanthus emblica*) with *Lactobacillus gasseri*: A nutraceutical remedial approach

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Abstract

Street food is an integral part of India's culinary culture, offering patrons a wide range of affordable options. However, concerns about the public's health risks associated with street food consumption persist due to inadequate infrastructure and the transient nature of street vendors. Contaminated street food can lead to foodborne illnesses such as gastroenteritis, typhoid fever, pneumonia, food poisoning, and hepatitis A. Probiotics, which are beneficial bacteria, are potentially effective by targeting specific infections. Probiotic-enriched fruit drinks present an innovative approach to improving population nutrition and probiotic provision. *Lactobacillus gasseri* is a gram-positive bacterium that has been demonstrated to be a promising strain of probiotic due to its resistance to acidity and beneficial effects on gut health. Alginate encapsulation improves the stability and release of probiotic strain. Probiotic fruit juices can be made from fruits that are rich in antioxidants and nutrients, such as *Passiflora ligularis*, *Fragaria ananassa*, and *Phyllanthus emblica*. The aim of this study was to use molecular methods to discover *Klebsiella aerogenes* (PP335235) isolated from street foods. The study also sought to determine the isolate's pattern of antibiotic sensitivity and explore the potential of fortifying fruit juice with *Lactobacillus gasseri* as a probiotic nutraceutical therapeutic strategy.

Keywords: Street food pathogens; Probiotics; *Lactobacillus gasseri*; *Klebsiella aerogenes*; Alginate encapsulation

1. Introduction

Street food is a significant and well-liked aspect of dining out in the India. It contributes to the region's rich culinary culture by providing a wide range of delectable and reasonably priced options [1]. In addition to being valued for their distinctive flavors, foods sold on the street present prospects for budding businesses [2]. The perception that eating street food poses a serious risk to public health due to a lack of basic infrastructure and services, the diversity, mobility, and transient nature is being believed by many people [3]. Even though many people enjoy street food, it's vital to be aware of any possible health dangers. Unregulated street food vendors sometimes harbor dangerous microorganisms like *E. coli* and *Salmonella*. If the food is not handled and prepared correctly, this can result in illness [4]. Consumers of street food have been linked to food-borne illnesses such as food poisoning, cholera, diarrhea, and typhoid fever [5].

1.1. Primitive diseases induced by street food

Consuming contaminated street food can result in a number of foodborne illnesses in India, as it does in many other places.

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1.1.1. Gastroenteritis

Inflammation of the stomach and intestines, known as gastroenteritis, is frequently brought on by eating or drinking food or water tainted with bacteria like *Salmonella*, *Escherichia coli* (*E. coli*), *campylobacter*, *shigella*, *Staphylococcus aureus*, *Yersinia*, *Clostridiodes difficile*, viruses like norovirus, or parasites. Diarrhea, cramping in the abdomen, nausea, and vomiting are other symptoms [6].

1.1.2. Food Poisoning

Food poisoning can result from eating street food that has been contaminated with dangerous microorganisms such as *Salmonella*, *Staphylococcus aureus*, or *Escherichia coli* (*E. coli*). Frequent symptoms include fever, diarrhea, vomiting, and stomach pain [6].

1.1.3. Typhoid fever

Consuming tainted food or water can lead to the bacterium *Salmonella typhi*, which causes typhoid fever. High fever, headache, and stomach pain are among the symptoms; in more severe cases, various organ issues may arise [6].

1.1.4. Hepatitis A

Hepatitis A is a highly contagious liver infection caused by the Hepatitis A virus. Yellowing of eyes and skin, dark urine, joint pain, clay-colored stools, fatigue, nausea and vomiting, loss of appetite, low-grade fever are the symptoms. Hepatitis A virus spreads easily through any contact with contaminated stool, contaminated food and water, close contact with an infected person such as shaking hands, sexual contact, both oral and anal, with an infected person and sharing needles or syringes [6]

1.1.5. Pneumonia

Pneumonia is an infection that inflames the air sacs in one or both lungs. It can be caused by a variety of organisms, including bacteria like *Klebsiella pneumoniae*, *Streptococcus pneumoniae*. Symptoms include chest pain when you breathe or cough, confusion or changes in mental awareness (in adults age 65 and older), cough, which may produce phlegm, fatigue, fever, sweating, and shaking chills, or diarrhea, shortness of breath [6].

1.2. View on probiotics over antibiotics

Afflictions instigated by foodborne pathogens can be effectively remediated through the judicious application of both antibiotics and probiotics. One of the most well-known types of chemotherapy in medical history is probably antibiotics. When antibiotics were first introduced in 1928, the rate of illness in humans fell sharply. With the discovery of antibiotics, the majority of diseases that were once thought to be fatal were now curable [7]. The rate of sickness increased with time. One of the main issues resulting from the increased use of antibiotics, and more specifically from their misuse in combinations and dosage level caused the emergence of antibiotic-resistant bacteria [8].

Probiotics, which are beneficial bacteria, offer a promising alternative to antibiotics in resolving issues caused by several street food pathogens. Unlike antibiotics, probiotics target specific pathogens while preserving the overall gut flora, thereby reducing the risk of antibiotic resistance and supporting quicker recovery from foodborne illnesses [9].

In order to counteract the drawbacks of antibiotics, the idea of probiotics was developed. Probiotics are live bacteria that give the host health benefits when given in sufficient doses. They bring the microbial ecosystem's regular, healthy equilibrium back [10].

1.3. Probiotic fortified fruit juice as remedy

Fortification is generally defined as the addition of one or more vital nutrients to food and raising the concentration in that food product to levels greater than normal [11]. Probiotic fortification involves the addition of live beneficial bacteria, such as *bifidobacterium* or *lactobacillus*. Probiotic bacteria are capable of enhancing the gut microbiota, preventing pathogenic infections, preventing or treating diarrhea, lowering cholesterol, boosting immunity [12]. Fruit juices enhanced with probiotics could be utilized as a vehicle for delivering probiotics and as a functional, wholesome beverage to support improved population nutrition and health. Probiotic fortification can be done by either way, direct addition of probiotic strain or probiotic fermentation [13].

1.4. Alginate based encapsulation of probiotic strain for better strain release

The potential application of encapsulated products is increased because encapsulation is a useful technique for substance protection and release control. It has been effectively applied to protect substances that are susceptible to heat, light, oxidation, moisture, and other undesirable reactions [14]. The inoculation of probiotic strain after encapsulating it provides better strain release. Even though the entrapment stress prevents the immobilized/encapsulated cells from growing, their stabilized growth during storage at 4 to 7 °C outlasted the free cells survival rate [15].

1.5. Probiotic strain

Gram-positive lactic acid bacteria generate lactic acid, are resistant to acid, and do not sporulate [16]. Several species of potentially probiotic microbes found are *Lacticasei bacillus reuteri*, *Lacticasei bacillus casei*, *Lacticasei bacillus casei LA1*, *Lacticasei bacillus rhamnosus GG*, *Lactiplanti bacillus plantarum 299*, and *Lactobacillus gasseri* are a few of them to have been used in a variety of therapeutic applications [17]. Among them, a native and indigenous human intestine LAB that is homofermentative, acid-resistant, and bile-tolerant is *Lactobacillus gasseri* [16]. Due to its ability to stick to intestinal tissues, activate macrophages, and create bacteriocins that can lower harmful microbes, it is commonly linked to probiotic effects. In the digestive system, *L. gasseri* may break down oxaloacetate while preserving serum and renal equilibrium [18].

Additionally, it has been noted that it can shield the intestinal barrier from inflammatory chemicals such lipopolysaccharides from diets high in fat, preventing their access into the bloodstream and thereby reducing inflammation in adipose tissue [19].

1.6. Fruits used to develop probiotic fruit juice

1.6.1. Phyllanthus emblica (Amla)

Of all the medicinal plants, *Phyllanthus emblica*, commonly called Indian gooseberry or Amla is known for its acidity and biopreservative nature. *P. emblica* is abundant in minerals, vitamins C, amino acids, tannins, phenolic compounds, and other nutrients [20].

1.6.2. Fragaria ananassa (Strawberry)

The *Fragaria ananassa*, known for its good antimicrobial activity. A low-calorie, fat-free, cholesterol-free, and sodium-free food that is rich in vitamins, fiber, and polyphenols in strawberries. Because fresh strawberries contain a lot of water, they are low in carbs [21].

1.6.3. Passiflora ligularis(Yellow passion fruit)

Passiflora fruit has a high quantity of phenolic and ascorbic acid, is low in sodium, produces no cholesterol, and is a very rich source of fiber and minerals, particularly potassium [22]. The antioxidant activity of passion fruit, which is linked to polyphenols particularly the flavonoids is its most researched biological function. Passion fruit is used extensively not only for eating raw but also for creating concentrates and juices in Brazil, one of the major producers and consumers of this fruit [23].

2. Materials and methods

2.1. Collection and incubation of food samples

Two different food samples of chicken fried rice were collected from two different street vendors near Gandhipuram Bus Stand in Coimbatore, India (Latitude-10.9289099 and Longitude-76.98446769999998). After purchasing the study was processed within 2-4 hours and used for further studies.

To initiate the isolation process, 0.2 g of Nutrient Broth was added to 15 ml of distilled water and was autoclaved at 121 °C for 15 minutes to create a sterile nutrient medium. Subsequently, each food sample was introduced into separate test tubes containing the Nutrient Broth and subjected to incubation for 90 minutes for potential growth.

2.2. Identification of microorganism

2.2.1. MacConkey agar

The media was prepared by dissolving 0.75 g in 15 ml of distilled water and was sterilized under an autoclave at 121 °C for 15 minutes. After sterilization the media was poured to sterilized petri plate and allowed for solidification. To the solidified media, one loop of culture was transferred using a wire loop and streaked on the plate to identify the bacteria. The streaked plate was incubated at 37 °C for 24 hours [24].

2.2.2. CLED(Cystine-Lactose-Electrolyte-Deficient) agar

The medium was prepared by dissolving 0.5 g in 15 ml of distilled water and was sterilized under an autoclave at 121 °C for 15 minutes. After that, the media was poured to the sterilized petri plate and allowed for solidification. To the solidified media, one loop of culture was transferred using a wire loop and streaked on the plate to identify the bacteria. The streaked plate was incubated at 37 °C for 24 hours [24].

2.3. Subculturing of bacteria

The obtained bacterial sample 1 taken from the CLED Agar plate was sub-cultured in Luria Bertani broth (Himedia, Mumbai, India) after sterilization. The media was prepared by dissolving 0.375 g in 15 ml of distilled water and sterilizing it under an autoclave at 121 °C for 15 minutes. Sterilized media was cooled to room temperature under aseptic conditions and transferred one loop of culture in a test tube and was incubated at 37 °C for 24 hours.

2.4. Antibiotic Susceptibility Pattern analysis using disc and hi comb assay

80µl of 24 hours old subculture was swabbed on agar plates of 75% of Mueller-Hinton agar. Antibiotics comb of N(A) and discs of CIP- Ciprofloxacin, CZ - Cefazolin, MET - Methicillin, AMP - Ampicillin, CAZ - Ceftazidime, VA- Vancomycin were placed on the plates and incubated for 24 hours at 37 °C and the zone of inhibition was measured in mm.

2.5. Molecular identification of bacteria

2.5.1. DNA isolation by ethanol precipitation technique

For the isolation of DNA, 1.5 µl of the bacterial culture was taken in an Eppendorf tube and was allowed to centrifuge for 5 minutes at 5000 rpm. To the pellet, 500 µl of 1x TE buffer and 20 µl of lysozyme was added. The mixture is incubated at 37 °C for 20 minutes. After incubation, 150 µl of 10% SDS was added and allowed to incubate in the water bath at 65 °C for 15 minutes. To the mixture, 180 µl of Phenol, 160 µl of Chloroform and 10 µl of isoamyl alcohol were added and centrifuged for 10 minutes at 10,000 rpm. Three different phases were formed. After pipetting out the first aqueous layer containing DNA, it was transferred into a new Eppendorf tube. 0.2 volume of Sodium acetate was added along with 1000 ml of Isopropanol and is centrifuged for 10 minutes at 12,000 rpm to achieve the thread like structure of DNA. The supernatant is discarded and to the pellet, 700 µl of 100% ethanol was added. The mixture was centrifuged at 5000 rpm for 5 minutes. After 15-20 minutes of drying, 40 µl of 1x TE buffer was added and stored in the refrigerator for further studies.

2.5.2. PCR amplification

The amplification of DNA was carried out in a reaction mixture with the final volume of 20 µl containing 1 µl of total DNA, 1 µl of each primer (SHV-F 5'-ACG GCT ACC TTA CGA CTT-3' and SHV-R 5'-GAG TTT GAT CCT GGC TCA-3'), 6 µl of PCR master mix, 4 µl of PCR buffer and 6 µl of distilled water. A gradient PCR was followed in the experiment.

The PCR reaction condition was as follows: 94 °C for 3 minutes, followed by 20 cycles of denaturation at 94 °C for 15 seconds, annealing at 53 °C - 55 °C for 15 seconds and extension at 72 °C for 2 minutes, before a final extension at 72 °C for 15 seconds for 20 cycles. The PCR product was analyzed using 0.8% agarose gel [25].

2.5.3. Agarose gel electrophoresis

Agarose powder (e.g., Merck) was dissolved in 1x TAE buffer and heated until the solution melted completely to create a 0.8% agarose gel. After melting, the mixture was added to the gel casting tray along with a comb to make wells and 2 µl of ethidium bromide, and left to solidify. After the gel solidified, it was carefully taken out of the comb and put into the electrophoresis unit. In the meantime, tracking dyes for visualization were created by combining DNA samples with loading dye in 1x TAE buffer. The gel's wells were then filled with the prepared samples. For 45–60 minutes, the electrophoresis was carried out at 50V to enable the negatively charged DNA fragments to move through the agarose

matrix and toward the positive electrode. Following gel electrophoresis, the gel was visualized under UV light to observe the separated DNA bands [26].

2.5.4. 16s rRNA sequencing and analysis with NCBI database

The product was subjected to partial 16S rRNA gene sequencing at Chromous Pvt Ltd in Bangalore using a genetic analyzer. The next step involved analyzing the sequenced data using the Basic Local Alignment Search Tool (BLAST) to determine which culture sequence in the National Center for Biotechnology Information (NCBI) database was the closest match for phylogenetic analysis. In addition, the sequenced gene was added to the NCBI database for future research and reference [27].

2.6. Collection of fruit samples and juice preparation

Fruits such as *Passiflora ligularis* - Yellow passion fruit, *Fragaria ananassa* – Strawberry, *Phyllanthus emblica* - Indian Gooseberry were procured from LuLu Hypermarket in Coimbatore, India. The fresh juice was formulated with the ratio of 2:2:1 (Strawberry:Yellow passion fruit:Amla) without adding any other ingredients and/or preservatives.

2.7. Preparation of production media and culturing of probiotic strain - *Lactobacillus gasseri*

The MRS broth was prepared by dissolving 1.103 g in 20 ml of the distilled water and sterilizing it under an autoclave at 121 °C for 15 minutes. Sterilized media was cooled to room temperature under aseptic conditions and the bacteria was transferred and was incubated at 37 °C for 24 to 48 hours. After getting the growth, this was used for further study [28].

2.8. Encapsulation of probiotic strain - *Lactobacillus gasseri*

A solution containing 3% sodium alginate and a different solution containing 2% calcium chloride were prepared and autoclaved for 15 minutes at 121 °C to sterilize them. The sodium alginate solution was mixed with *Lactobacillus gasseri* (*L.gasseri*) suspended in it to make a homogeneous mixture. To help the $CaCl_2$ solution get into the microcapsules, this mixture was added dropwise using a micropipette onto a petri plate. The plate was then incubated for two hours to allow the formation of the calcium alginate beads that were encasing *L.gasseri*. The final product was obtained by drying the sample after it had been incubated and filtering it to remove the microcapsules from any excess solution. *L.gasseri* was found in the microcapsules produced by this process, which may find use in probiotic delivery systems, among other applications [29].

2.9. Preparation of fortified juices with probiotic pellets

The encapsulated probiotic strain was added in different formulations and allowed to ferment at room temperature.

The formulations as follows:

- Control (Pure juice mixture without the pellets)
- Fruit juice + 20 pellets of the probiotic strain
- Fruit juice + 50 pellets of the probiotic strain

2.10. Total phenolic content of pure fruit extracts and fortified probiotic fruit juices - Folin-Ciocalteu method

Total phenolic content of the sample was studied with the Folin–Ciocalteu reagent method with slight modifications. To the 1 ml of extract, 0.2 ml of 10% Folin–Ciocalteu's reagent was added, mixed by gentle shaking and kept for 5 minutes in room temperature, followed by addition of 1 ml of Na_2CO_3 (7%, w/v) and incubated at 30 °C for 30 minutes. The absorbance of the sample was recorded at 765 nm using a UV-vis spectrophotometer (Labtronics LT291, Microprocessor) [30].

2.11. Total antioxidant test of pure fruit extracts and fortified probiotic fruit juices - Phosphomolybdenum method

Using phosphomolybdenum method, total antioxidant activity was confirmed by the protocol of Phatakand Hendre (2014) and Priest *et al.,* (1999) with slight modification. 0.5 ml of the sample was mixed with the 0.5 ml of reaction mixture containing 0.6 M H_2SO_4, 28 mM sodium phosphate and 4mM ammonium molybdate reagent. The solution was incubated at 50 °C for 90 minutes with blank. After incubation the tubes were normalized into room temperature and the absorbance was read at 695 nm using a spectrophotometer (LT 291 labtronics microprocessor) [31].

2.12. Nutrient analysis

2.12.1. Carbohydrate estimation - Anthrone test

0.5 ml of sample was mixed with 2.5 ml of the anthrone reagent and allowed to stand in boiling water bath for 10 minutes at 40 °C. Cooled the sample to room temperature and colour developed was read at 620 nm in the spectrophotometer [32].

2.12.2. Protein estimation - lowry et al., method

Pipette out 0.5 ml of the sample extract in a test tube and added 2.5 ml of Solution C (Solution A- 2% Na_2CO_3 in 0.1 N NaOH and Solution B- 0.5% $CuSO_4$ solution, Solution C= 50:1 ratio of Solution A and B.). After mixing the tube was allowed to stand for 10 minutes, added 0.2 ml of Folin's Ciocalteu reagent, mixed well and incubated at room temperature in the dark condition for 30 minutes. After incubation the OD was read at 660 nm using a spectrophotometer [33].

2.13. Test for presence of enteric pathogens in the fortified juices

The fortified probiotic fruit juices were used for the identification of presence of pathogens. MacConkey agar was prepared by dissolving 0.74 g in 15 ml of the distilled water and sterilized under an autoclave at 121 °C for 15 minutes. Sterilized media was poured to the petri plate and allowed for the solidification, to the solidified media all the treated sample was used to streak separately by single streaking method and was incubated at 37 °C for 24 to 48 hours.

2.14. Test for viability of probiotic strain in the fortified juices

Viability of the sample was tested with MRS agar. The media was prepared by dissolving 0.82 g in 15 ml of the distilled water and sterilized under an autoclave at 121 °C for 15 minutes. Sterilized media was cooled to room temperature under aseptic conditions and transferred to a petri plate and both Juice + 20 pellets and Juice + 50 pellets were poured to the petri plate and incubated at 37 °C for 24 hours.

2.15. Antibacterial activity using well diffusion method

The agar well diffusion method was used to study the antibacterial activity of the sample. Nutrient broth medium was used to subculture the bacteria and was incubated at 37 °C for 24 hours, afterwards, 20µl cultures of *Streptococcus aureus, Klebsiella aerogenes* and *Salmonella typhi* were taken and spread on the Mueller hinton agar plates (38 g of Mueller hinton agar in 1000 ml of distilled water and sterilized under autoclave at 121 °C for 15 minutes) to cultivate a uniform microbial growth plates. Followed by wells were made with cork borer and the samples were added to the respective wells along with negative control (DMSO) and Antibiotic disc of C30 as positive control. Finally, the petri dishes were incubated for 24 hours at 37 °C. In order to evaluate the antibacterial activity of the samples, the diameter of the inhibition zone was measured and noted [34].

2.16. Minimum Inhibitory Concentration study - Elisa 96 plate assay

This method was initiated using sterile 96-well plates [35]. The wells of each row were filled with 1 µl sterilized nutrient broth. Sequentially, samples (Fruit Juices) in different concentrations of 5 µl, 10 µl, 15 µl ,20 µl and 25 µl along with 10 µl of the culture, *Klebsiella aerogenes*. The wells were incubated for 24 hours at 37 °C. The resulting turbidity was observed, after 24 hours MIC was determined to be where growth was no longer visible by assessment of turbidity by optical density readings at 600 nm with a 96 well ELISA plate reader (Robonik). % of cell death was calculated using the formula,

$$\% \text{ of cell death } = \frac{\text{Control OD } - \text{ Sample OD}}{\text{Control OD}} \text{x } 100$$

3. Results and discussion

3.1. Collection and incubation of food samples

The signs of microbial growth such as turbidity, gas production and changes in color were observed.

Figure 1 Incubated Food samples

3.2. Identification of microorganism

3.2.1. MacConkey agar

The growth of colonies of different unknown microorganisms were seen in the MacConkey agar plate. The sample 1 exhibited white color whereas the sample 2 exhibited reddish pink color.

Figure 2 Bacterial growth on MacConkey Agar

3.2.2. CLED(Cystine-Lactose-Electrolyte-Deficient) agar

The growth of colonies of different unknown microorganisms were seen in the CLED agar plate. The sample 1 exhibited white color whereas the sample 2 exhibited reddish pink color.

Figure 3 Bacterial growth on CLED Agar

3.3. Subculturing of bacteria

The signs of microbial growth such as turbidity, gas production and changes in color were observed.

3.4. Antibiotic Susceptibility Pattern analysis using disc and hi comb assay

The size of the zones of inhibition surrounding antibiotic discs or the color shift in the HiComb assay are used to interpret the results. Greater zones of inhibition suggest that the bacteria are more susceptible to the tested drug.

Figure 4 ASP Using antibiotics discs

Figure 5 ASP Using comb (N(A))

Table 1 Zone measurement (in mm) for antibiotic discs

Name of the antibiotics	Zone measurements (in mm)
CIP- Ciprofloxacin	9
CZ - Cefazolin	No clear zone formation
MET - Methicillin	No zone formation
AMP - Ampicillin	No clear zone formation
CAZ - Ceftazidime	No clear zone formation
VA- Vancomycin	2

Description : Ciprofloxacin (CIP) showed a zone diameter of 9 mm and moderate sensitivity. But there was no clear zone formation, indicating resistance, for Cefazolin (CZ), Methicillin (MET), or Ceftazidime (CAZ). Vancomycin (VA) showed a small zone of inhibition, indicating that it was only moderately effective against the bacteria under study.

Table 2 Zone measurement (in mm) for antibiotic comb

Concentration of the antibiotic in comb	Zone measurements (in mm)
0.01	0
0.1	0
5	1
10	1
30	1
90	1
120	2
240	2

Description: These results represented that the inhibition zones are probably influenced by the comb concentration, with higher concentrations leading to larger zones of inhibition formation.

3.5. Molecular identification of bacteria

3.5.1. DNA isolation by ethanol precipitation technique

After pipetting out the aqueous phase, both Sodium acetate and Isopropanol initiated a network like structure of DNA.

The isolation of bacterial DNA using the ethanol precipitation technique resulted in a purified DNA pellet, which was collected after much centrifugation. This pellet contains the extracted DNA, ready for further analysis.

3.5.2. PCR amplification

After performing PCR (Polymerase Chain Reaction) to amplify the gene, gel electrophoresis is typically conducted to confirm successful amplification. The PCR product can be submitted to a sequencing facility for 16S rRNA gene sequencing.

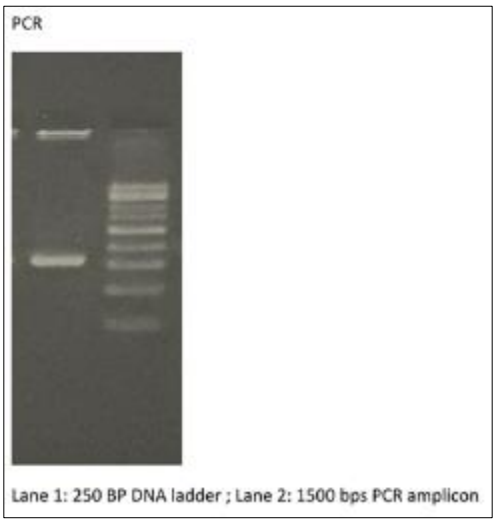

Figure 6 PCR Amplicon

3.5.3. Agarose gel electrophoresis

A distinct band corresponding to different-sized DNA fragments were identified after loading a sample of DNA fragments acquired through PCR amplification.

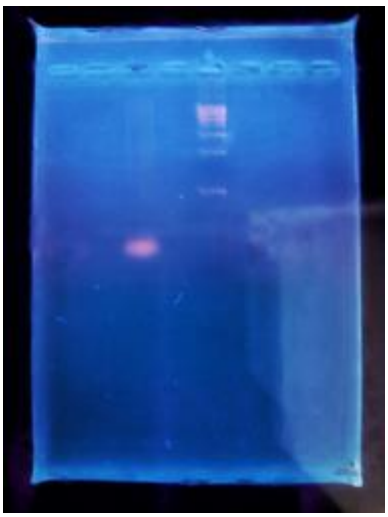

Figure 7 DNA fragments in gel

3.5.4. 16s rRNA sequencing and analysis with NCBI database

Once sequencing is completed, the obtained sequences are analyzed, and the results are compared to existing databases such as NCBI (National Center for Biotechnology Information) to identify the bacterial species present in the sample.

Additionally, the obtained sequences were submitted to the NCBI GenBank database for deposition and public access and were found to be *Klebsiella aerogenes*, strain PHBK (PP335235). This submission includes providing relevant metadata about the sequences and their associated experimental details.

The submitted the sequence is given below in the FASTA format,

>PP335235.1 *Klebsiella aerogenes* strain PHDK 16S ribosomal RNA gene, partial sequence

TGCAGTCGACCCTTAGCACAGAGAGCGTGCTCTAGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGG

AAACTGCCTGATGGAGGGGGATAACTCCTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAG

TGGGGGACCCTCGGGCCTCATGCCATCAGATGTGCCAGATGGGATTAGCTAGTAGGTGGGGTAATGGCTC

ACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGAC

TCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTAT

GAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGAGGAGGAAGGCGTTAAGGTTAATAACCTTAGCGAT

TGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGC

GTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCT

CAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGC

GGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCA

GGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTG

GAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGC

AAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAA

CGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTAGCAGAGATGCTTTGGTGCCTTCGGGAACTC

TGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGC

AACCCTTATCCTTTGTTGCCAGCGGTCCGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAG

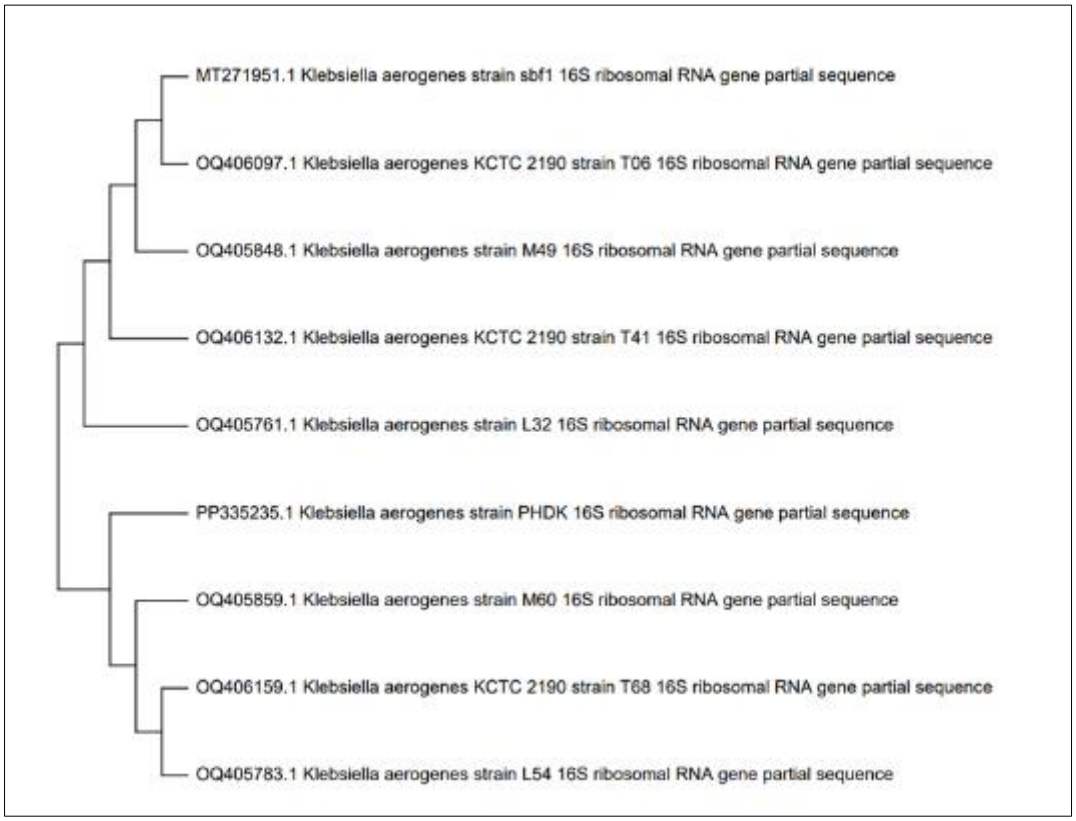

Figure 8 The phylogenetic tree was constructed using MEGA 11 software.

3.6. Collection of fruit samples and juice preparation

Figure 9 Prepared fruit juice mixture

The prepared fruit juice mixture was stored at room temperature for further studies.

3.7. Preparation of production media and culturing of probiotic strain - *Lactobacillus gasseri*

The result indicated successful growth of *Lactobacillus gasseri*.

3.8. Encapsulation of probiotic strain - *Lactobacillus gasseri*

Capsules were formed on the surface of the $CaCl_2$ solution. The capsules were filtered and dried for further studies.

Figure 10 Dried probiotic pellets

3.9. Preparation of fortified juices with probiotics pellets

The encapsulated *L.gasseri* was added as 20 pellets and 50 pellets separately to proceed further studies.

Figure 11 Prepared fortified probiotic fruit juice mixtures

3.10. Total phenolic content of pure fruit extracts and fortified probiotic fruit juices - Folin-Ciocalteu method

Concentrations of phenolic compounds are higher when the OD values are higher.

Table 3 Total phenol content of **pure fruit extracts** at 765 nm

SAMPLE	**OD**
Blank	0
Passiflora ligularis	0.1219
Fragaria ananassa	0.846
Phyllanthus emblica	0.734

Description : The total phenol concentration of raw juice samples, measured at a wavelength of 765 nm, is shown in the table above. According to the data, *Passiflora ligularis* had the greatest phenol concentration, whereas *Phyllanthus emblica* had the lowest phenol content.

Table 4 Total phenol content of fortified juices at 765 nm

Sample	18 Hours	7 Days	14 Days
Blank	0	0	0
Control	0.1252	0.742	0.766
20 Pellets+Fruit juice	1.591	0.688	0.765
50 Pellets+Fruit juice	1.675	0.765	0.854

Description : The total phenol concentration of the different juice samples, measured at a wavelength of 765 nm, is shown in the table above. According to the data, control had the greatest phenol concentration after 18 hours, 7 days and 14 days of the juice preparation, whereas 50 Pellets+Fruit juice had the lowest phenol content.

3.11. Total antioxidant test of pure fruit extracts and fortified probiotic fruit juices - Phosphomolybdenum method

The overall antioxidant capacity of the fruit extract increases with an increase in absorbance value. Thus, differences in absorbance over time would be expected for each fruit extract, showing variances in antioxidant capacity. Greater absorbance values correspond to increased antioxidant activity, whereas lower values reflect a gradual decline in antioxidant activity.

Table 5 Total antioxidant content of pure fruit extracts at 695 nm

SAMPLE	OD
Blank	0
Passiflora ligularis	0.634
Fragaria ananassa	0.786
Phyllanthus emblica	0.465

Description : The total antioxidant concentration of raw juice samples, measured at a wavelength of 695 nm, is shown in the table above. According to the data, *Fragaria ananassa* had the greatest antioxidant concentration, whereas *Phyllanthus emblica* had the lowest antioxidant content.

Table 6 Total antioxidant content of fortified juices at 695 nm

SAMPLE	18 HOURS	7 DAYS	14 DAYS
Blank	0	0	0
Control	0.241	0.621	0.467
20 Pellets+Fruit juice	0.286	0.547	0.382
50 Pellets+Fruit juice	1.339	0.629	0.238

Description : The total antioxidant concentration of the different juice samples, measured at a wavelength of 695 nm, is shown in the table above. According to the data, 50 Pellets+Fruit juice had the greatest antioxidant concentration after 18 hours and 7 days of the juice preparation whereas control had the greatest antioxidant concentration after 14 days.

3.12. Nutrient analysis

3.12.1. Carbohydrate estimation - Anthrone test

The overall carbohydrate capacity of the fruit extract increases with an increase in absorbance value. Thus, differences in absorbance over time would be expected for each fruit extract, showing variances in carbohydrate capacity. Greater absorbance values correspond to increased carbohydrate content, whereas lower values reflect a gradual decline in carbohydrate content.

Table 7 Carbohydrate estimation at 620 nm

Sample	OD
Blank	0
Control	0.301
20 Pellets+Fruit juice	0.418
50 Pellets+Fruit juice	0.468

Description: The carbohydrate content of raw juice samples, measured at a wavelength of 620 nm, is shown in the table above. According to the data, 50 Pellets + Fruit juice had the greatest carbohydrate content, whereas control had the lowest carbohydrate content.

3.12.2. Protein estimation - lowry et al., method

The overall protein capacity of the fruit extract increases with an increase in absorbance value. Thus, differences in absorbance over time would be expected for each fruit extract, showing variances in protein capacity. Greater absorbance values correspond to increased protein content, whereas lower values reflect a gradual decline in protein content.

Table 8 Protein estimation at 660 nm

SAMPLE	OD
Blank	0
Control	0.504
20 Pellets+Fruit juice	0.667
50 Pellets+Fruit juice	0.712

Description : The protein content of Probiotic juice samples, measured at a wavelength of 660 nm, is shown in the table above. According to the data, 50 Pellets + Fruit juice had the greatest protein content, whereas control had the lowest carbohydrate content.

3.13. Test for presence of enteric pathogen of the fortified sample

The plate showed zero presence of enteric pathogens in both Juice + 20 pellets and Juice + 50 pellets.

Figure 12 Test for enteric pathogens

3.14. Test for viability of the probiotic strain in the fortified juices

The results of viability are as follows:

- After 7 days - Juice + 20 pellets showed maximum amount of viability
- Juice + 50 pellets showed maximum amount of viability

Figure 13 Presence of *Lactobacillus gasseri*

3.15. Antibacterial activity using well diffusion method

Used the agar well diffusion method, the antibacterial activity of three different samples was evaluated. The possible antibacterial activity of the samples against *Salmonella typhi*, *Klebsiella aerogenes*, and *Streptococcus aureus* was indicated by the observation of inhibition zones surrounding the wells.

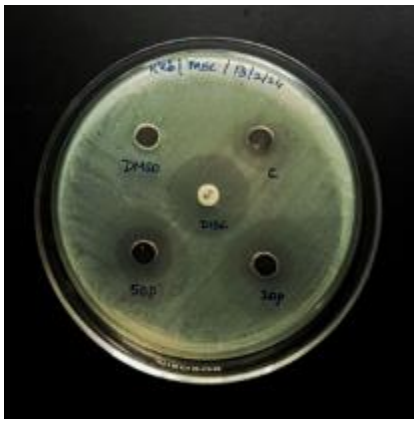

Figure 14 Antibacterial activity of *Klebsiella aerogenes*

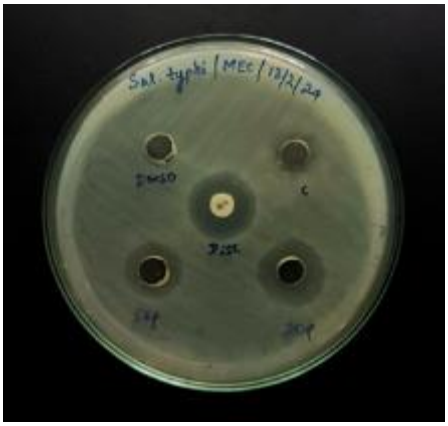

Figure 15 Antibacterial activity of *Salmonella typhi*

Figure 16 Antibacterial activity of *Staphylococcus aureus*

Table 9 Zone of Inhibition (mm)

Name of the organism	Control	Juice+50 pellets	Juice+20 pellets	DISC	DMSO
Klebsiella aerogenes	17	25	26	23	Nil
Staphylococcus aureus	15	15	17	17	Nil
Salmonella typhi	16	17	22	20	Nil

Description : Juice and pellet combination exhibits higher inhibition when compared to DMSO and control. While *Staphylococcus aureus* shows constant inhibition across treatments, *Klebsiella aerogenes* shows significant inhibition with both pellet concentrations. *Salmonella typhi* exhibits significant inhibition with 20 pellets + juice. These findings, which show differences between bacterial strains and pellet concentrations, point to a possible antibacterial effect of the juice-pellet combination.

3.16. Minimum Inhibitory Concentration study - Elisa 96 plate assay

The Minimum Inhibitory Concentration(MIC) assay result shows the smallest concentration of a material required to stop a microorganism from growing.

Table 10 Minimum Inhibitory Concentration Study

Concentrations of *K. aerogenes* culture	**Blank**	**Control**	**Juice+20 Pellets**	**Juice+50 Pellets**
5 µl	0	0.530	12.45	50
10 µl	0	0.530	26.03	51.69
15 µl	0	0.530	48.11	68.49
20 µl	0	0.530	72.83	73.39
25 µl	0	0.530	73.01	78.67

Description : The MIC values drop with increasing juice and pellet concentration, suggesting a more potent inhibitory effect on the organism. The highest % of cell death is seen in Juice + 50 pellets proving that the probiotic juice acts as antimicrobial resistant agent.

4. Conclusion

This research work included the isolation and molecular identification of the street food bacteria by Polymerase Chain Reaction (PCR) and 16s rRNA sequencing. The sequenced data is then submitted in the National Centre for Biotechnology Information (NCBI) as a database. The identified bacteria (*Klebsiella aerogenes*, PP335235) was then annotated.

The nutraceutical remedial approach involved fortifying probiotic fruit juices, where in the probiotic strain (*Lactobacillus gasseri*) was encapsulated rather than directly added in order to better strain release and enhanced shelf life. The probiotic fruit juice formulation comprised a blend of fruits such as *Fragaria ananassa*, *Passiflora ligularis*, and *Phyllanthus emblica* and fermented with encapsulated probiotic strain (*Lactobacillus gasseri*). This formulation showed the resistivity to the *Klebsiella aerogenes* and some common street food pathogens such as *Staphylococcus aureus* and *Salmonella typhi,* as hypothesized to be efficacious in treating diseases attributed to Enterobacteriaceae family. Additionally, the fortified juice underwent various assessments such as MIC, DNA Damage study, and Shelf life assessment of the encapsulated probiotic strain.

With the development of fortified probiotic fruit juices fermented with encapsulated *Lactobacillus gasseri*, which exhibit resistivity to common street food pathogens like *Klebsiella aerogenes, Staphylococcus aureus,* and *Salmonella typhi*. This work not only contributes to regulatory compliance by providing sequenced data to databases like NCBI for better oversight of food safety standards, but it also offers a potential therapeutic approach with incorporation of probiotics for diseases linked to infections within the Enterobacteriaceae family.

Compliance with ethical standards

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Disclosure of conflict of interest

No conflict of interest to be disclosed.

References

[1] Abrar, S.M., Mohammedsani, Z.S. Street Food Consumption and Associated Health Risk. 2020; 6(7): 8-18.

[2] Sharmila, R. Street Vended Food in Developing World:Hazard Analyses. Indian J Microbiol, 2011;51(1): 100–106.

[3] deSousa, C.P. The impact of food manufacturing practices on food borne diseases. Food Science and Technology-Braz. arch. biol. Technol. 2008; 51(4): 815-823.

[4] Reda, N., Ketema, B., Tsige, K. Microbiological quality and safety of some-street-vended foods in Jimma Town, Southwestern Ethiopia, African Journal of Microbiology Research, 2017; 11(14): 574-585.

[5] Kaul, M., Agarwal, G. Microbial load of common chat products, 1998; 25(6): 197-199.

[6] Anderson, A.A., Aline, D.P., Alessandra, B.F.M. Microbiology of street food: understanding risks to improve safety applied Microbiology. 2023; 8(134): 1-11.

[7] Aminov, R.I. A brief history of the antibiotic era: lessons learned and challenges for the future. Frontiers in microbiology. 2010; 134(1): 1-7.

[8] Mithra, N.H., Mrinalini. Antibiotics And Probiotics : A Brief Review. Central India Journal of Dental Sciences, 2021; 9(2): 35-43.

[9] William, W., Ze Xiang., Xi, Y., Ping, L., Hao, C., Mingfei, Y., Yiqiu, F., /Yilun, H., Yeshi, Y., Hang, X. The Challenge of Applications of Probiotics in Gastrointestinal Diseases. Advanced Gut & Microbiome Research,2023; 1(2023): 1-10.

[10] Divya, P. Benefits of Probiotics in Oral Cavity – A Detailed Review. Annals of International Medical and Dental Research, 2016; 2(5): 10-17.

[11] Bouis, H.E., Hotz, C., McClafferty, B., Meenakshi, J.V., Pfeiffer, W.H. Biofortification: A new tool to reduceterization of mixed pineapple and hibiscus jam. Revista Agro@mbiente On-line. 2011; 1(32): 155–163.

[12] Singh, N., Madhu. Scientific and Technical Aspect of Functional Probiotic Yogurt Fortification: a Review. IJCRT, 2018;6(2): 2320-2882.

[13] Ravinder, N., Ashwani, K., Manoj, K. Fortification and fermentation of fruit juices with probiotic lactobacilli. Annals of Microbiology, 2012;1(62): 1573–1578.

[14] Faria, M., Hajek, A.E., Wraight, S.P. Imbibitional damage in conidia of the entomopathogenic fungi *Beauveria bassiana, Metarhizium acridum,* and *Metarhizium anisopliae*. Biol Contr. 2009; 51(3): 346–354.

[15] Darko, D., Elena, V., Tomaz, L., Eleonora, W. Apple juice as a medium for fermentation by the probiotic *Lactobacillus plantarum* PCS 26 strain. Annals of Microbiology. 2015; 1(65): 2161–2170.

[16] Mahboubi, M. *Lactobacillus gasseri* as a functional food and its role in obesity. Int. J. Med. Rev, 2019; 6(2): 59–64.

[17] Zommiti, M., Feuilloley, M.G.J., Connil, N. Update of probiotics in human world: A nonstop source of benefactions till the end of time. Microorganisms, 2020; 8(12): 1-30.

[18] Selle, K., Klaenhammer, R.T. Genomic and phenotypic evidence for probiotic influences of *Lactobacillus gasseri* on human health. FEMS Microbiol. Rev, 2013; 37(6): 915–935.

[19] Kawano, M., Miyoshi, M., Ogawa, A., Sakai, F., Kadooka, Y. *Lactobacillus gasseri* SBT2055 inhibits adipose tissue inflammation and intestinal permeability in mice fed a high-fat diet. J. Nutr. Sci, 2016; 1(5): 1-9.

[20] Calixto, J. B., Santos,A. R., Cechinel,F. V., Yunes, R. A. A review of the plants of the genus Phyllanthus: their chemistry, pharmacology, and therapeutic potential. Med Res Rev, 1998; 18(1): 225-258.

[21] Finn, C. E., Retamales, J. B., Lobos, G. A., Hancock, J. F. The Chilean strawberry (*Fragaria chiloensis*): Over 1000 years of domestication. HortScience. 2013; 48(4): 418–421.

[22] Sandi, D., Chaves, J.B.P., Sousa, A.C.G., Parreiras, J.F.M., Silva, M.T.C., Constant, P.B.L. Hunter colour dimension, sugar content and volatile compounds in pasteurized yellow passion fruit juice (*P. edulis var flavicarpa*) during storage. Brazilian Archives of Biology and Technology, 2004; 47(1): 233–254.

[23] Heim, K. E., Tagliaferro, A. R., Bobilya, D. J. Flavonoid antioxidants: Chemistry, metabolism and structure-activity relationships. The Journal of Nutritional Biochemistry, 2002; 13(10): 572-584.

[24] Tessa, S., Eddy, B.W., and Wiwiek, T. Isolation and Identification of Escherichia coli O157:H7 Lytic Bacteriophage from Environment Sewage. International Journal of Food Science, 2021; 1(5): 1-11.

[25] Atawodi, S.E., Atawodi, J.C., Dzikwi, A.A. Polymerase chain reaction: Theory, practice and application: A review. Sahel Medical Journal, 2010; 13(2): 54-63.

[26] Queipo, M I., Colmenero, J.D., and Macias, M., Bravo, M.J., Morata, P. Preparation of Bacterial DNA Template by Boiling and Effect of Immunoglobulin G as an Inhibitor in Real-Time PCR for Serum, 2008; 15(2): 293-296.

[27] Altschul, W.F., Gish, W., Miller, E.W., and Lipman, D.J. Basic local Alignment search tool. Journal of Molecular Biology. 1990; 215(3): 403–410.

[28] Zanjani, M.K., Tarzi, B.G., Sharifan, A., Mohammad,i N, Bakhoda, H., Madanipour, M.M. Microencapsulation of *Lactobacillus casei* with calcium alginate-resistant starch and evaluation of survival and sensory properties in cream-filled cake. Agricultural and Food Sciences, 2012; 6(26): 5511-5517.

[29] Favaro, T.C., Grosso, C. Microencapsulation of *L. acidophilus* (La-05) and *B. lactis* (Bb-12) and evaluation of their survival at the pH values of the stomach and in bile. J Microencapsul, 2002; 19(4): 485-494.

[30] Singleton, V. L., Rossi, J. A. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents.The American Journal of Enology and Viticulture, 1965; 16(4): 144–158.

[31] Jaya Prakash, M.A., Raghunathan, R., Jesteena, J. Evaluation of bioactive compounds from *Jasminum polyanthum* and its medicinal properties. Journal of drug delivery and therapeutics, 2019; 9(2): 303-310.

[32] David, T., Plummer, C. An Introduction to Practical Biochemistry. Internet Archive Books. 1990; 179(3): 294-299.

[33] Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. Protein measurement with the folin phenol reagent. J Biol Chem, 1951; 193(1): 265-275.

[34] Jesteena, J., Radhai, S., Raghunatha, R. Extraction of Chitin and Chitosan from Wild Type Pleurotus Spp and its Potential Application - Innovative Approach. Journal of Pure and Applied Microbiology, 2018; 12(3): 1631-1640.

[35] Wiegand, I., Hilpert, K., Hancock, R.E.W. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nature Protocols, 2008; 3(2): 163–175.