FORMULATION OF FERMENTED PROBIOTIC *Punica granatum* JUICE AND ITS ACTIVITY AGAINST GIT INFECTION CAUSING ORGANISMS

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ABSTRACT

In this study, production of probiotic pomegranate juice through its fermentation by lactic acid bacteria was determined. The pomegranate juice was fermented with Lactobacillus sps isolated from curd using MRS medium. Biochemical tests were done to confirm that the isolated organism belongs to Lactobacillus sps. Viable cell was determined by the standard plate count method using MRS medium. Culture capable of forming $1.3x10^7$CFU/mL was inoculated into the juice and left undisturbed for the process of fermentation to occur. Fermentation was carried out at 27^oC for 48 hours. The nutrient content (carbohydrate, protein, iron content, vitamin C) of the fermented pomegranate juice was measured and compared with fresh pomegranate juice and there was a significant increase in the nutritional value .The antimicrobial activity of the juice was also observed against gastrointestinal tract (GIT) infections. Citric acid as a major organic acid in pomegranate juice was significantly consumed by all probiotic lactic acid bacteria, thus Pomegranate juice was proved to be a suitable media for production of a fermented probiotic drink, especially as a probiotic supplement for vegans.

Keywords: *Lactobacillus sp*, pomegranate, fermentation, probiotic, gastrointestinal tract infections(GIT), antimicrobial activity, nutritional value.

1.INTRODUCTION

Probiotics are defined as alive microbial supplement, which beneficially affect the host when administrated in adequate amounts by improving its intestinal microbial balance. Research has shown that probiotic bacteria can colonize and proliferate in the intestinal tract of humans and animals to prevent the growth of intestinal pathogens (Ishibashi et al.,2005).Multiple reports have described their health benefits on gastrointestinal infections, antimicrobial activity, improvement in lactose metabolism, reduction in serum cholesterol, immune system stimulation, anti-diarrheal properties, improvement in inflammatory bowel disease, enhancing mucosal barriers, synthesizing different types of vitamin B, antimutagenic properties, anti-carcinogenic properties (Hashemi et al., 2017).Moreover, it is generally believed that the minimum concentration of living probiotic microorganism in the product (probiotic contained) at the time of consumption should be at least 10^7 CFU/mL to achieve the proposed health benefits.

Probiotics have been added to yogurt and other fermented dairy products. However, an increased demand for non-dairy probiotic products comes from vegetarianism due to milk cholesterol content, milk allergy and others factors (Ray & Sivakumar, 2009). This fact has led to development of probiotic products from various food matrices including fruits and vegetables. Fruits and vegetables have been suggested as an ideal media for probiotic growth because they inherently contain essential nutrients like vitamins, minerals, sugars, which supports the growth of probiotics (Sheehan et al., 2007). There is a genuine interest in the development of fruit juice based functional beverages with probiotics because they have taste profiles that are appealing to all age groups and because they are perceived as healthy and refreshing foods.

Pomegranate (*Punica granatum*, Punicaceae) is known to have considerable health-promoting properties with antimicrobial, antiviral, anticancer, antioxidant and anti-mutagenic effects, improves haemoglobin level (Negi et al.. 2003). The fresh juice contains 85.4% water and considerable amounts of total soluble solids (TSS), total sugars, reducing sugars, antho-cyanins, phenolics, ascorbic acid and proteins and has been reported to be a rich source of antioxidants. These antioxidants are more potent, on a molar basis, than many other antioxidants including vitamin C, vitamin E, coenzyme Q-10 and alpha-lipoic acid. According to (Mousavi et al. 2013), fermentation of pomegranate juice reduced total anthocyanin content. In this context, the use of probiotic bacteria is a useful strategy to obtain products with longer shelf life as well as safer properties due to their ability to delay or preventing the growth of common contaminant bacteria.

The crucial roles of probiotic such as lactic acid bacteria (LAB) in maintaining the human gastrointestinal tract (GIT) microbial ecosystem by the growth inhibition of ingested pathogens were well demonstrated (Servin et al., 2014). Lactobacillus strains also can be used in improving of food products due to their probiotic properties as well as fermentative activities (Yousefabad,et al 2016). Due to this, the pomegranate juice was fermented with the *Lactobacillus sp* isolated from curd using MRS medium and the culture capable of forming 1.3x 10^7 CFU/mL was inoculated into the juice and left undisturbed for the process of fermentation to occur. After fermentation for 48hrs at room temperature(27^o C), the nutritional value for carbohydrate, protein, vitamin C, iron were compared with fresh juice and antimicrobial property was identified.

Most of the time, infections of the gastrointestinal tract result in diarrhoea or dysentery, nausea, vomiting, and abdominal cramping which result as a cause of ingestion and infection. Bacteria which are associated with GIT infections are *E.coli, Salmonella sp, Staphylococcus aureus, Klebsiella sp , and Bacillus ss, etc.*Among the various microbiota that colonize the human gut, Gram-negative bacteria have been most notoriously linked to GIT-related diseases such as inflammatory bowel disease (IBD) including Crohn's disease and ulcerative colitis and colorectal cancer (CRC).

E.coli infection by ingesting (taking in by mouth) certain strains of *E. coli* bacteria. The bacteria travel down the digestive tract, releases a destructive toxin, called the Shiga toxin, which damages the lining of the small intestine. The growing infection causes symptoms like bloody diarrhoea. However, studies in recent years are beginning to recognize a new

player, *Klebsiella pneumonia* in the causation and progression of GIT diseases. Once synonymous with infections and diseases of the upper respiratory tract, *K. pneumoniae* has now emerged as one of the pathogens commonly isolated from patients with GIT diseases. However, extensive studies attributing *K. pneumoniae* to GIT diseases, particularly that of CRC are scanty. *Klebsiella quasipneumoniae* is an opportunistic pathogen causing antibiotic-resistant infections of the gastrointestinal tract in many clinical cases.
Staphylococcal enteritis is an inflammation that is usually caused by eating or drinking substances contaminated with staph enterotoxin by *Staphylococcus aureus*. This causes inflammation leading to abdominal pain, cramping, dehydration, diarrhoea and fever. Pseudomonas infections can infect any part of your body, such as the blood, lungs, stomach.*Pseudomonas aeruginosa* is an ubiquitous gram-negative opportunistic human pathogen which is not considered part of the human commensal gut microbiota. However, depletion of the intestinal microbiota (Dysbiosis) following antibiotic treatment facilitates the colonization of the intestinal tract by Multidrug-Resistant Pseudomonas. It is a major cause of nosocomial infection.

Therefore, the current study was designed to produce a non-dairy probiotic pomegranate juice by fermentation for 48hrs at 27^{o}C using *Lactobacillus* strain from curd especially for vegans, possessing inherent health benefits and to check if there is any the enhancement in nutritional value and its antimicrobial properties against GIT infections.

2.MATERIALS REQUIRED

2.1.GLASSWARES AND INSTRUMENTS:

- Petri dishes
- Conical flasks
- Test tubes
- Inoculation loop
- Incubator
- Autoclave
- Hot air oven
- Centrifuge
- Well borer
- Pipette
- Beaker

2.2.BACTERIAL STRAINS:

In this study clinical isolated strains of *E.coli, S.aureus, Pseudomonas sp, Klebsiella sp*. The culture was procured from the KMCH Hospital situated in SITRA, Coimbatore.

2.3.CULTURE MEDIA& SUBSTRATE:

- Substrate- Pomegranate juice
- Sample- Curd
- MRS agar (as shown in Table.1)
- MRS broth (as shown in Table.2)

- Muller-Hinton agar (as shown in Table.3)

COMPOSITION OF CULTURE MEDIA:

- **MRS AGAR MEDIA:**

S.NO	CHEMICAL	CONCENTRATION (g/l)
1.	Peptone	5g
2.	Beef extract	3g
3.	Sodium chloride	5g
4.	Glucose	2g
5.	Sodium acetate trihydrate	0.5g
6.	Polysorbate	0.1g
7.	Di potassium hydrogen phosphate	0.2g
8.	Tri ammonium citrate	0.2g
9.	Magnesium sulfate heptahydrate	0.02g
10.	Agar-agar	20g

Table (1). Composition of MRS agar

- **MRS BROTH:**

S.NO	CHEMICAL	CONCENTRATION (g/l)
1.	Peptone	5g
2.	Beef extract	3g
3.	Sodium chloride	5g
4.	Glucose	2g
5.	Sodium acetate trihydrate	0.5g
6.	Polysorbate	0.1g
7.	Di potassium hydrogen phosphate	0.2g
8.	Tri ammonium citrate	0.2g
9.	Magnesium sulfate heptahydrate	0.02g

Table (2). Composition of MRS broth

- **MULLER-HINTON AGAR:**

S.NO	CHEMICAL	CONCENTARION(g/l)
1.	Beef extract	2.00gm
2.	Acid hydrolysate of casein	17.50gm
3.	Starch	1.50gm
4.	Agar	17.00gm
5.	Distilled water	1000ml

Table (3). Composition Muller-Hinton agar

3. METHODOLOGY

3.1.SAMPLE COLLECTION:

Lactobacillus are primary predominantly present in curd, milk, yogurt. The curd sample was brought from the store and it was transferred to a sterile screw cap test tube.

3.2.ISOLATION OF *Lactobacillus:*

3.2.1.SERIALDILUTION AND SPREAD PLATE:

From the curd sample, 1 ml was taken and mixed well in a test tube containing 10ml of distilled water. A series of culture tubes containing 9ml of sterile water were taken. From the stock culture, 1ml suspension was transferred aseptically to the 1st tube (10^{-1}), mixed well. From the 1st tube, 1ml of suspension was transferred into the 2nd tube (10^{-2}), mixed well. The same process was then repeated for the remaining tubes and taken 1ml from the last tube and dispend it out. Dilution up to 10^{-6} were made .From the serial dilution of $10^{-3,}10^{-4,}10^{-5}$, 1 ml was spread plated on the MRS agar plates. The diluted samples were evenly spread using a sterile L- rod. The plates are labelled by its specific dilution factor and kept for incubation at 37°C for 48 hours.

3.2.2.STREAKING:

To get isolated colonies of the specified microorganism, the colonies were selected based on colony colour, sliminess and viscosity. The colonies were purified by repeated streaking on MRS agar plates. In refrigerated condition the pure culture was stored both in sterile water and in slants.

3.2.3.IDENTIFICATION AND CONFIRMATION OF ISOLATED *Lactobacillus:*

Morphological, cultural and biochemical characters were considered for the characterization and identification of *Lactobacillus.*

1. CULTURAL CHARACTERIZATION:

For colonial characteristics the colonies were purified and cultured on the following media:

- **MRS AGAR:**

MRS is the selective media for the growth of *Lactobacillus.* MRS agar media was prepared, sterilized and inoculated with the isolated colonies of *Lactobacillus.* The inoculated plates were incubated at 37°C for 24 hours. After incubation, the plates were observed for cultural characterization.

2. MORPHOLOGICAL CHARACTERIZATION:

For morphological characteristics and gram reaction Gram staining was performed.

Gram staining was performed and observe through microscope.

3. BIOCHEMICAL CHARACTERIZATION:

For the identification of the microorganism many biochemical tests were performed. The tests include:

1. Indole production test
2. Methyl red test
3. Voges Proskauer test
4. Citrate utilization test
5. Urease test
6. Catalase test
7. Oxidase test
8. Carbohydrate fermentation test

The media for all thesetests were inoculated aseptically. The incubation period for the inoculated media was 24-48 hours at 37°C.

3.3.FERMENTATION OF POMEGRANATE JUICE:

Fresh pomegranate juice was prepared from clean and fresh pomegranates without any presence for contamination or damage and the juice was pasteurised at 80^{o} C for 5mins. 10mL of the cultured MRS broth was taken and centrifuged at 5000rpm for 8mins and the pellets of *Lactobacillus* were inoculated aseptically to 100ml of the juice.It was allowed to ferment for 48 hours at room temperature as shown in Fig.(1)

Fig (1). Fermentation of pomegranate juice

3.4.ANALYSIS OF NUTRITIONAL CONTENT:

After the completion of the fermentation process, the following nutrient contents were analysed and quantified

1. CARBOHYDRATE
2. PROTEIN
3. IRON
4. VITAMIN C

These values were also compared with the nutrient contents of fresh pomegranate juice.

3.5. ANALYSIS OF ANTI-MICROBIAL ACTIVITY:

Muller Hinton agar was prepared and sterilized in an autoclave at 121^{o}c for around 20 mins. The media was poured in sterile Petri dishes and allowed to solidify. After the media was solidified two wells were cut using a well borer in MHA plates. The plates were

inoculated with cultures of *E. coli, Klebsiellasp, Staphylococcus aureus and Pseudomonas sp*. One well was added with 150µl of the sample and another well was added with 200µl of the sample. The plates were inoculated in the incubator at 37^0c for 24 to 48 hours and plates were observed for zone of clearance.

4.RESULTS

4.1.COLLECTION OF SAMPLE AND ISOLATION:

Growth was observed in the MRS agar plated with the dilutions $10^{-3,}10^{-4,}10^{-5}$of the sample. From the spread plate separate colonies were streaked on to and isolated using MRS agar medium and separate individual colonies were observed.

Fig (2). Colonies of *Lactobacillus* sps MRS medium

4.2.MORPHOLOGICAL CHARACTERIZATION OF *Lactobacillus:*

When Gram stained, gram positive purplecoloured rods were observed under 40x and 100x (oil immersion) objectives of compound microscope.

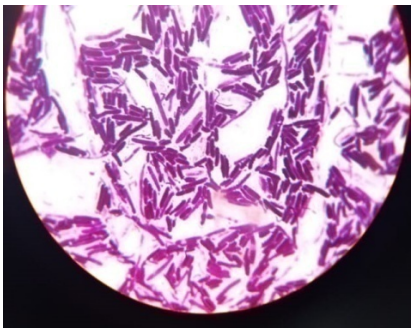

Fig (3). Gram staining of *Lactobacillus* under 100 X (oil immersion)

4.3.BIOCHEMICAL CHARACTERIZATION OF *Lactobacillus:*

TEST	RESULT
Indole production test	Negative
Methyl red test	Positive
Voges Proskauer	Negative
Citrate utilisation test	Negative
Urease test	Negative
Catalase test	Negative
Oxidase test	Negative
Glucose fermentation	Positive (no gas formation)
Lactose fermentation	Positive (no gas formation)
Sucrose fermentation	Positive (no gas formation)

Table (4). Biochemical test results

4.4.FERMENTATION OF POMEGRANATE JUICE:

The pomegranate juice was fermented for 48 hours at room temperature.

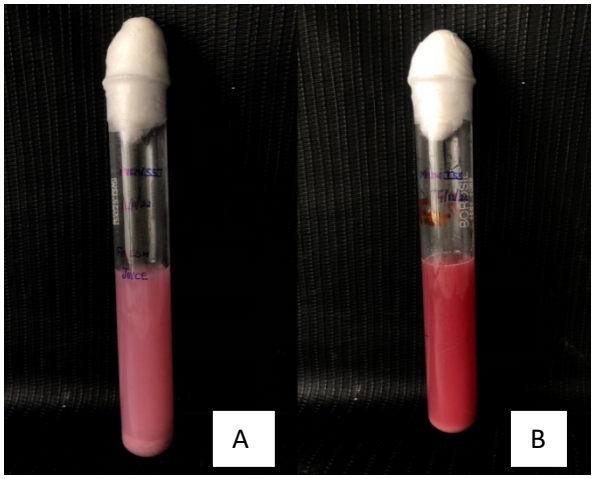

Fig (4). A (fresh juice) &B (fermented pomegranate juice)

4.5. ANALYSIS OF NUTRITIONAL VALUE :

The nutritional value for the probiotic pomegranate juice and fresh pomegranate juice was compared. The following nutrient values were taken into consideration while comparing:

1. CARBOHYDRATE
2. PROTEIN
3. IRON
4. VITAMIN C

NUTRITION	FRESH JUICE	FERMENTED JUICE
Carbohydrate	25.0gm	27.46gm
Protein	1.67gm	2.5gm
Iron	4%	4.7%
Vitamin C	12mg	14mg

Table (5). Comparison of nutrient values

4.6.ANTIMICROBIAL ACTIVITY:

The antimicrobial activity of the fermented pomegranate juice was also observed against certain pathogenic organisms which are

1. *Staphylococcus aureus*
2. *Escherichia coli*
3. *Klebsiella sp*
4. *Pseudomonas sp*

S.NO	ORGANISM	ZONE OF CLEARANCE	
		150µl	200µl
1	*S. aureus*	13.0mm	14.0mm
2	*E. coli*	12.0mm	18.0mm
3	*Pseudomonas sp*	-	5.0mm
4	*Klebsiella sp*	-	10.0mm

Table (6). Estimation of zone of clearance

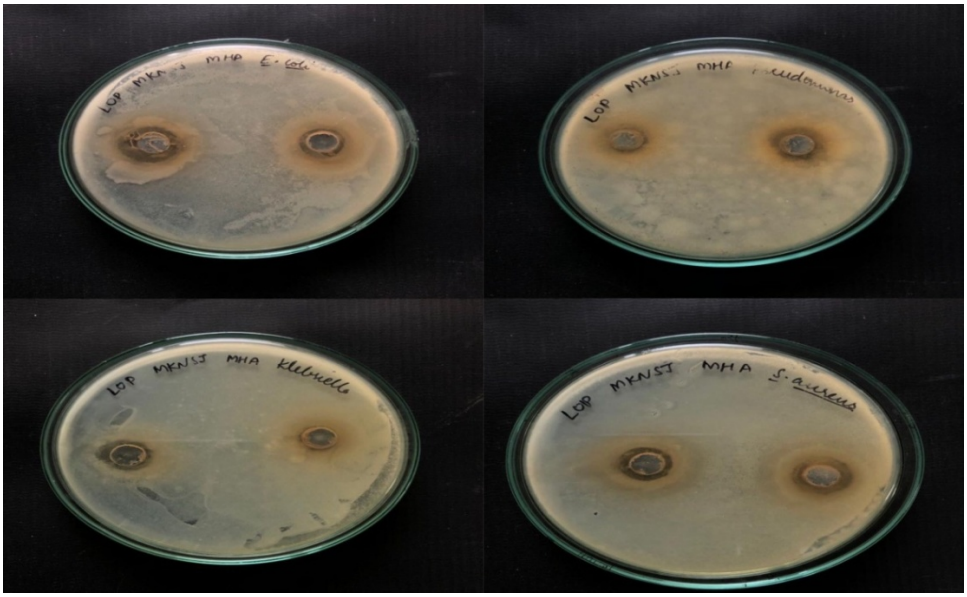

Fig (5). Antimicrobial activity against (left to right) *E. coli, Pseudomonas sp, Klebsiella sp, S. aureus*

5.CONCLUSION AND DISCUSSION

Lactobacilli are one of the most common probiotics used in variety of products such as yogurts, curd, cheese. These organisms are capable of enhancing the nutrient content of various food products and it also improves the human gut microflora by inhibiting the growth of pathogenic bacteria. In this study *Lactobacillu*s was isolated from curd sample. Pomegranate juice was fermented with *Lactobacillus* culture and after fermentation the nutritional value of it was compared to fresh pomegranate juice, the fermented one had comparatively high nutrient content due to presence of probiotic in them which indicates nutritional enhancement. The antimicrobial activity of the juice was also observed and measured by the formation of zone of clearance against certain pathogens namely against *S. aureus, E. coli, Klebsiella sp, Pseudomonas sp*. Many previous studies have shown that pomegranate juice has anti-inflammatory, anti-oxidative, and antimicrobial activity of which the antimicrobial activity was proven in this study. Therefore, the fermented pomegranate juice can be further developed and consumed as a non-dairy probiotic source as a juice formulation especially by vegans,which possess value added dual benefits of enhanced nutritional quality and also as a source of probiotic to maintain the gut microbiota.Future studies can be focused on analysing the other beneficiary properties of the juice such as anti-inflammatory and anti-oxidant properties. The fermented juice can also be carried forward and made into a commercial product so that it can be made available in the market.

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