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ISOLATION AND CHARACTERIZATION OF LACTIC ACID BACTERIA AND THEIR ROLE IN INHIBITING PATHOGENS OF URINARY TRACT INFECTIONS

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Abstract:

Lactic acid bacteria are widely distributed in the nature. They can be isolated from soil, water, plants, silage, from the intestinal tract of animals and humans and fermented food products. Lactic Acid Bacteria (LAB) are characterized as Gram positive, usually non-motile, non-sporulating bacteria that produce lactic acid as a major or sole product of fermentative metabolism. Lactic acid bacteria are among the best studied microorganisms for human health advantageous effects and fermentation. Significant novel developments have been made on lactic acid bacteria in the area of multidrug resistance, bacteriocins, osmoregulation, autolysins and bacteriophages. Different lactobacilli, bifidobacteria, or probiotic mixtures have been shown to alleviate digestive diseases in experimental animals and in inflammatory bowel diseases in humans. Antimicrobial activities of LAB have been demonstrated in various species. Some LAB can prevent the adherence, establishment and invasion or toxin production of intestinal or vaginal pathogens. Health benefits currently being investigated in favour of probiotic microorganisms include their role in alleviating chronic intestinal inflammatory diseases; prevention and treatment of pathogen induced diarrhoea; urogenital infections and atopic diseases.

The present study investigates the inhibitory activity of LAB against the pathogens of urinary tract infection. The LAB used in this study were isolated from plant sources along with use of curd to isolate LAB.The LAB were isolated and characterised and their inhibitory activity was checked against the pathogens of urinary tract infection. LAB showed a greater range of inhibitory activity than observed for antibiotics commonly used against the pathogens.

Key Words: Lactic Acid Bacteria, Urinary Tract Infections, Pathogens, Bacteriocins, Inhibition

INTRODUCTION:

Lactic acid bacteria are widely distributed in the nature. They can be isolated from soil, water, plants, silage, from the intestinal tract of animals and humans and fermented food products. Lactic Acid Bacteria (LAB) are characterized as Gram positive, usually non-motile, non-sporulating bacteria that produce lactic acid as a major or sole product of fermentative metabolism (Choksi N. *et al.* 2012).

Most species have multiple requirements of amino acids and vitamins. Because of this, lactic acid bacteria are generally abundant only in habitats where these requirements are met. They are often associated with animal oral cavities and intestines; plant leaves as well as decaying plants or animal matter, compost, etc. The classification of lactic acid bacteria into different genera is largely based on morphology, mode of glucose fermentation, and configuration of the lactic acid produced. Lactic acid bacteria are among the best studied microorganisms for human health advantageous effects and fermentation. Significant novel developments have been made on lactic acid bacteria in the area of multidrug resistance, bacteriocins, osmoregulation, autolysins and bacteriophages. Advancement has also been made in the production of food grade genetically modified lactic acid bacteria (Choksi N. *et al.* 2012).

The lactic acid bacteria can be mainly divided into two groups based on the end-products formed during the fermentation of glucose. Homofermentative lactic acid bacteria such as *Pediococcus, Streptococcus, Lactococcus* and some lactobacilli produce lactic acid as the major or sole end-product of glucose fermentation.

Homofermentative lactic acid bacteria use the Embden-Meyerhof-Parnas pathway to generate two moles of lactate per mole of glucose and derive approximately twice as much energy per mole of glucose as heterofermentative lactic acid bacteria. Heterofermentative lactic acid bacteria such as *Weissella* and *Leuconostoc* and some lactobacilli produce equimolar amounts of lactate, CO2 and ethanol from glucose via the hexose monophosphate or pentose pathway (Rattanachaikunsopon P. *et al.* 2010).

Strains of lactic acid bacteria, used as starter culture, may inhibit spoilage microorganisms and pathogens by production of metabolites with anti-microbial properties. Different lactobacilli, bifidobacteria, or probiotic mixtures have been shown to alleviate digestive diseases in experimental animals and in inflammatory bowel diseases in humans.

Antimicrobial activities of LAB have been demonstrated in various species. Moreover, their antagonistic actions are demonstrated against numerous intestinal and food-borne pathogens, such as *Escherichia coli O157:H7, Clostridium difficile, Listeria monocytogenes, Staphylococcus aureus* and several others. Some LAB can prevent the adherence, establishment and invasion or toxin production of intestinal or vaginal pathogens. They can also inhibit the growth of pathogenic bacteria by a pH reduction through the production of organic acids such as acetic, propionic or lactic acid, or by producing hydrogen peroxide. LAB can also compete for nutrients or adhesion site against pathogens. One important attribute of lactic acid bacteria is their ability to produce antimicrobial compounds such as organic acids, diacetyl, hydrogen peroxide, ethanol, reuterin and bacteriocins or bactericidal proteins ((Millette M. *et al.* 2006 Ogbonna P. *et al.* 2014).

Bacteriocins produced by lactic acid bacteria are defined as extracellular primary or modified products of bacterial ribosomal synthesis, which can have relatively narrow spectrum of bactericidal activity. Health benefits currently being investigated in favour of probiotic microorganisms include their role in alleviating chronic intestinal inflammatory diseases; prevention and treatment of pathogen induced diarrhoea; urogenital infections and atopic diseases. Bacteriocin producing strains can be used as starter cultures for fermented foods in order to improve safety and manage health more effectively (Ogbonna P. *et al.* 2014).

Structural analysis has confirmed that some of these bacteriocins are entirely different peptides, others can be breakdown products or oxidized forms of one bacteriocin. The antimicrobial activity of the bacteriocin is generally due to the action of a single peptide (Tahara T. *et al.* 1996).

The recent studies demonstrate that LAB bacteriocins share a common mechanism of action, which is the dissipation of the proton motive force (PMF) in target cells. The majority of bacteriocins produced by *L. acidophilus* are heat stable, low-molecular-mass, nonlantibiotic peptides which belong to class II on the basis of the recent classification for LAB bacteriocins (Tahara T. *et al.* 1996).

The bacteriocins produced by LAB have been reported to infiltrate the outer membrane of Gram-negative bacteria and to encourage the inactivation of Gram-negative bacteria in combination with other enhancing antimicrobial environmental factors, such as organic acid, low temperature and detergents materials. Bacteriocins are generally named based on the genus or species of the strain producing it. For example, *L. plantarum* produce plantaricin, Lactococcus spp. (lacticin, nisin), and Carnobacterium spp. (carnocin), Enterococcus spp (enterocin). Leuconostoc spp. (leucocin) Pediococcus spp. Pediocin (Yusuf M A. *et al.* 2013).

It seems that much of the renewed interest in these substances is a direct response to the perceived potential practical applications of these agents either to preservation of foods or to the prevention and treatment of bacterial infections (Patel S. *et al.* 2015).

The urinary tract is the second commonest site of bacterial infection and is a cause of significant morbidity both in terms of the number of people affected and the potential complications. Urinary tract infections

(UTI's) can be classified into complicated or uncomplicated UTI's. The traditional approach to treatment of lower UTI was 7 to 14 days of therapy but studies suggest that shorter three-day courses are as effective.

MATERIALS AND METHODS

Collection of samples:

Urine samples were aseptically collected into sterile bottles from Godbole Pathology Laboratory, Pune,Maharashtra,India and were immediately taken to the laboratory.(Michael L. Wilson 2004)

Culture and identification of pathogens:

Urine samples were centrifuged and checked for pus cells. These samples were inoculated in MacConkey's broth for enrichment and were incubated on shaker for 48 hrs. After observing growth in broth samples were plated on EMB agar, Citrate agar, MacConkey's agar respectively to get different isolated pathogens. Isolated colonies were then identified by cultural and biochemical tests.

Enrichment of lactic acid bacteria:

For lactic acid bacteria fruit peels, flower peels, curd sample were used as enlisted below:(Md. Ibrahim Khalil, 2017), (Yi-sheng Chen 2009) (S.C. Ribeiro 2013)

1. *Asparagus racemosus* Leaves
2. *Phyllanthus niruri* Leaves and fruits
3. *Cocos nucifera* Coconut cream
4. *Mangifera indica,* Mango peel
5. *Ocimum tenuiflorum* Leaves
6. *Zingiber officinale* Stem
7. *Catharanthus roseus* Leaves and Flowers
8. Curd sample

The samples listed above were immersed in Phosphate buffer solution for 2 hours and then transferred to MRS broth and incubated. After incubation for 2-4 days growth from the broth was streaked on MRS medium. Isolated colonies were then identified by cultural and biochemical tests.

Characterisation of Lactic Acid Bacteria:

pH

Each of the isolates of Lactic acid bacteria were inoculated in MRS broth of pH 4,7,11 respectively.
O.D. was measured between time intervals of 24, 48, 72 hours respectively.

Temperature

Each of the isolates of Lactic acid bacteria were inoculated in MRS broth which were exposed to three different temperatures of 24ºC, 37º C, 15ºC respectively. O.D. was measured between time intervals of 24, 48, 72 hours respectively.

Salt Concentration

Each of the isolates of Lactic acid bacteria were inoculated in MRS broth with salt concentration of 0.1gm%,0.25g% and 1gm%.
All the tubes were incubated at room temperature in dessicator.
O.D. was measured between time intervals of 24, 48, 72 hours.

Identification of pathogens

The isolated pathogens were identified using morphological, cultural and biochemical tests.

Effect of antibiotics on pathogens of urinary tract infection

The agar diffusion technique was used for determination of MIC in solid media. It involved the application of antibiotic solutions of different concentrations to paper discs, placed on the surface of or agar plates seeded with the test bacterial strain.

Antibiotic diffusion from these discs into the agar medium leads to inhibition of bacterial growth in the vicinity of the source and to the formation of clear 'zones'. The diameter of these zones increases with antibiotic concentration. (Boyan Bonev et al.2008)(Heshmatipour Z 2015)

After incubation at 37^0C for 24 hours zones of inhibition were measured in mm.

The antibiotics used were Amoxylin, Ceftriax, Ampicillin and Amikacin which are generally used to treat urinary tract infections.

Antimicrobial Assay:

Agar overlay method was used for detection of antimicrobial activity of lactic acid bacteria against pathogens isolated from infected urine samples.

The antibacterial activity of LAB isolates was determined by agar overlay method. LAB isolates were streaked on MRS medium in two parallel streaks. After incubating for 24-48 hours and checking for growth of LAB, Nutrient Agar medium was overlaid and allowed to solidify. Then culture of the test organism, (*Pathogen*), was spread on the nutrient agar and incubated for 24-48 h. Zone of inhibition was observed and antibacterial activity confirmed.

RESULTS AND DISCUSSION:

All the isolates obtained in this study were considered LAB, based on their positive Gram reaction, non-motility, absence of catalase activity, no spore formation, and the rod or coccus shape (George et al 2018).

Table1: Biochemical Tests for LAB isolates

Test	**Source of Lactic Acid Bacteria**							
	Ocimum tenuliforum	*Zingiber officinale*	Curd	*Asparagus racemosus*	*Phyllanthus niruri*	*Mangifera indica,*	*Cocos nucifera*	*Catharanthus roseus*
Glucose	+	+	+	-	+	+	+	+
Maltose	+	+	+	-	+	+	+	+
Sucrose	+	+	+	-	+	+	+	+
Lactose	+	+	+	-	+	+	+	+
Oxidase	-	-	-	-	-	-	-	-
Catalase	-	-	-	-	-	-	-	-
Indole	-	-	-	-	-	-	+	+
Methyl Red	+	+	+	+	+	-	-	-
Voges Proskauer	-	-	-	-	-	-	-	-
Citrate Utilisation	-	-	-	-	-	-	-	-

Gram Character	+	+	+	+	+	+	+	+

Table 2: Biochemical Tests for pathogens

Biochemical	Pathogen *E.coli*	*E.coli*	*Pseudomonas*	*Staphylococcus*	*Klebsiella*
Glucose	A+G	A+G	A+G	A+G	A+G
Maltose	A+G	A+G	A+G	A+G	A+G
Sucrose	A+G	A+G	A+G	A+G	A+G
Lactose	A+G	A+G	A+G	A+G	A+G
Oxidase	+	+	-	+	+
Catalase	+	+	+		+
Indole	+	+	+	-	-
Methyl red	+	+	+	+	+
Vogus –	-	-	-	-	-
Citrate	-	-	+	-	+
Gram's	-	-	-	+	-

Table 3: Effect of pH on growth of Lactic Acid Bacteria

Source of Lactic acid bacteria	24 hours O.D.			48 hours O.D.			72 hours O.D.		
pH	**4**	**7**	**9**	**4**	**7**	**9**	**4**	**7**	**9**
curd	0.05	0.17	0.65	0.09	0.61	0.81	0.10	1.24	1.15
Asparagus racemosus	0.05	0.62	0.51	0.08	0.69	0.77	0.55	1.28	0.88
Phyllanthus niruri	0.03	0.57	0.71	0.09	0.58	0.67	0.08	0.66	0.77
Cocos nucifera	0.07	1.21	0.45	0.06	1.22	1.29	0.05	1.19	1.24
Catharanthus roseus	0.05	1.14	0.74	0.09	1.15	0.92	0.07	1.30	1.17
Mangifera indica,	0.05	0.81	0.64	0.05	0.72	0.77	0.07	0.86	0.82
Ocimum tenuiflorum	0.05	0.82	0.73	0.07	0.83	0.74	0.08	1.10	0.80
Zingiber officinale	0.06	0.60	0.74	0.08	0.91	0.74	0.11	1.13	0.85

Table 4: Effect of Temperature on growth of Lactic Acid Bacteria

Source of Lactic acid bacteria	**24 hours**			**48 hours**			**72 hours**		
Temperarture	**37^{0}C**	**15^{0}C**	**R.T.**	**37^{0}C**	**15^{0}C**	**R.T.**	**37^0**	**15^{0C}**	**R.T.**
Curd	0.84	0.07	0.66	1.39	0.22	1.36	1.12	0.50	1.20
Asparagus racemosus	0.67	0.09	0.66	1.22	0.18	1.20	1.02	0.17	1.07

Phyllanthus Niruri	0.59	0.10	0.64	1.07	0.59	0.98	0.75	0.50	0.62
Cocos Nucifera	0.84	0.03	0.93	1.51	0.21	1.42	1.17	0.45	1.12
Catharanthus roseus	0.96	0.06	1.02	1.50	0.14	1.44	1.19	0.09	1.24
Mangifera indica	0.67	0.08	0.78	1.15	0.15	1.17	0.78	0.62	0.99
Ocimum tenuiflorum	0.66	0.05	0.71	1.10	0.39	1.17	0.78	0.62	0.99
Zingiber officinale	0.65	0.05	0.62	1.16	0.14	1.17	0.97	0.18	0.99

Table 5: Effect of salt concentration on growth of Lactic Acid Bacteria

Source of Lactic	**24 hours O.D.**			**48 hours O.D.**			**72 hours O.D.**		
Salt concentration	**0.1gm**	**1gm**	**0.25gm**	**0.1gm**	**1gm**	**0.25gm**	**0.1gm**	**1gm**	**0.25gm**
Curd	0.87	0.63	0.61	1.09	1.05	1.16	1.07	0.94	1.16
Asparagus	0.43	0.47	0.60	0.97	0.90	0.93	1.01	0.99	1.01
Phyllanthus	0.57	0.41	0.34	0.64	0.69	0.70	0.71	0.83	0.74
Cocos	0.13	0.98	0.97	1.21	1.09	1.08	1.33	1.29	1.20
Catharanthus	1.03	1.10	1.10	1.23	1.12	1.17	0.74	1.20	1.10
Mangifera	0.66	0.70	0.70	0.78	0.77	0.83	0.89	0.94	0.92
Ocimum	0.67	0.58	0.68	0.90	0.80	0.91	1.12	1.23	1.31
Zingiber officinale	0.56	0.49	0.54	1.06	1.04	1.03	1.19	1.13	1.18

Table 6: Effects of antibiotics on pathogens

Antibiotics	Amoxylin	Ceftriax	Ampicillin	Amikacin
E.coli 1	-	-	-	21 mm
E.coli 2	-	-	-	21mm
Staphylococcus spp.	19mm	16mm	-	20mm
Pseudomonas spp.	-	-	-	-
Klebsiella spp.	-	7mm	-	27mm

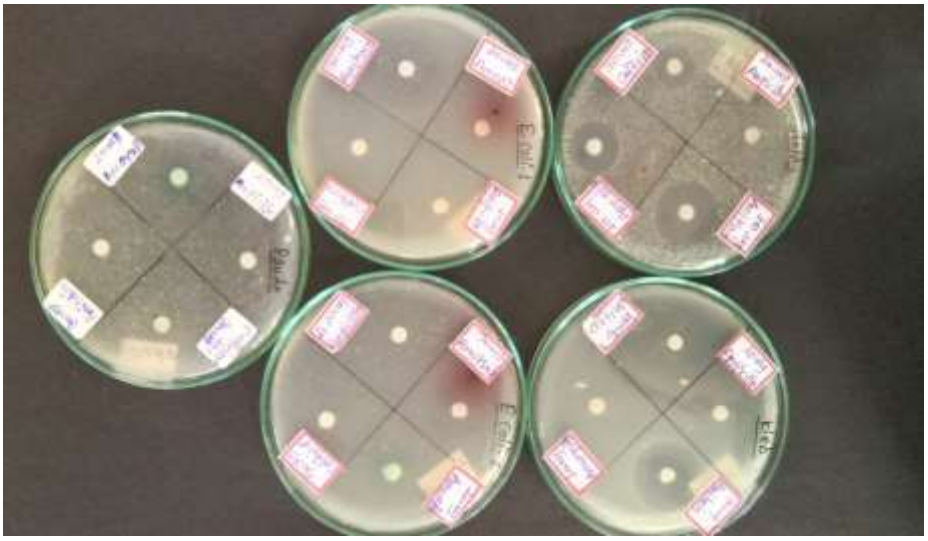

Fig1: Effects of antibiotics on pathogens

Table7: Antibacterial activity of isolates of Lactic acid Bacteria

Source of lactic acid	*E.coli 1*	*E.coli2*	*Pseudomonas*	*Staphylococcus spp.*	*Klebsiella*
Ocimum tenuiflorum	24mm	20mm	30mm+	15mm	10mm
Zingiber officinale	40-	20mm	22mm	17mm	15mm
Curd	40-	19mm	20mm	14mm	23mm
Asparagus racemosus	-	-	-	-	-
Catharanthus roseus	45mm	27mm	32mm	27mm	16mm
Cocos nucifera	30mm	15mm	23mm	24mm	12mm
Mangifera indica	35mm	22mm	31mm	19mm	5mm

Fig 2: Inhibitory activity of Lactic acid bacteria against pathogens of urinary tract infection

All bacteria were isolated on MRS agar plate which is a selective media for lactic acid bacteria. Colony characters were observed and Gram's nature was identified. Sugar fermentation tests showed different results for every isolate. All isolates were catalase negative. All isolates were oxidase negative. Indole, Voges Proskauer and citrate utilization test were negative for all isolates. Methyl red test was positive for all isolates.

All the pathogens fermented glucose, maltose, sucrose, lactose. All pathogens showed oxidase positive except *Pseudomonas aeruginosa.* Also, they have shown catalase test positive. Indole and Voges Proskauer tests were negative and methyl red and citrate utilization were positive for *Staphylococcus spp.* Different pathogens showed varying results for IMViC tests indicating their varying abilities.

As per the result it can be concluded that, LAB have shown effective growth at pH 9 at room temperature as compared to pH 7 and pH 4. It indicates that these bacteria are more likely to grow in alkaline conditions. This pH is higher than the observed range of pH for the growth of most of the lactic acid bacteria which falls between 5.8 to 8.3 (En Yang et al,2018).

As per the results, LAB grow at room temperature instead of 37^0C and 15^0C.This is in line with the temperature optimums observed for growth of most of the LAB (En Yang et al,2018). Also, as most of these LAB are plant isolates optimum growth at room temperature is most obvious.

The isolates of lactic acid bacteria have shown maximum growth at 0.1gm% salt concentration. This salt concentration is lower than that observed for growth of most Lactic isolates (Alžbeta Medved'ová,2018).

Amoxicillin/clavulanate, Cefdinir and Cephalexin are the most commonly used antibiotics to treat urinary tract infections. The side effects of most of these antibiotics include nausea, diarrhoea, vomiting, heartburn, stomach pain, rectal or genital itching. Dizziness and extreme tiredness. Though the antibiotics are effective in treating the infection the side effects may persist resulting in suffering of the patient for prolonged periods.

Also slowly the pathogens develop resistance to these antibiotics and hence higher antibiotics are needed to be administered which may increase the severity of the side effects and deteriorate the condition further.

As observed from Table 6, the antibiotics are effective against some pathogens where as other pathogens seem to be resistant to these antibiotics. As against this the LAB show a very good inhibitory activity against the pathogens which is very well demonstrated in Table7.

Except for the LAB isolate from *Asparagus racemosus* all other LAB isolates show a very good antibacterial activity. Also, the zones of inhibition observed are much larger than those obtained for the antibiotics. Probiotics control intestinal pathogens by production of antibacterial compounds, including lactic and acetic acid and antibiotic like substances, competition for nutrients and adhesion sites, increased and decreased enzyme activity, increased antibody levels and increased macrophage activity observed for the antibiotics (S. Hudault et al,1997). They are known to modulate the host immune response and microenvironment, such that risk of infection is reduced. The possibility of treatment of urinary tract infection by natural human probiotics with least side effects is achievable (In Seok Lim et al ,2008). It has been demonstrated that LAB has a high potential for the treatment of UTI. This is a viable option for chemotherapy of UTI (B.A. Adeniyi et al,2006). We are facing a future in which combination therapy for UTI treatment will be routine, as resistance rates to single agents rise to unacceptable levels worldwide and untreatable UTIs present a real concern. Alternatives to antibiotics for the treatment, augmentation of treatment or prevention of recurrent UTI are attractive options to reduce the risks of antimicrobial resistance (Néha Sihra et al ,2018).

CONCLUSION:

The study very well indicates the effectiveness of LAB to treat the urinary tract infections. The detection and analysis of compounds active in this action will help to further explore LAB to be used as alternatives to conventional antibiotics.

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