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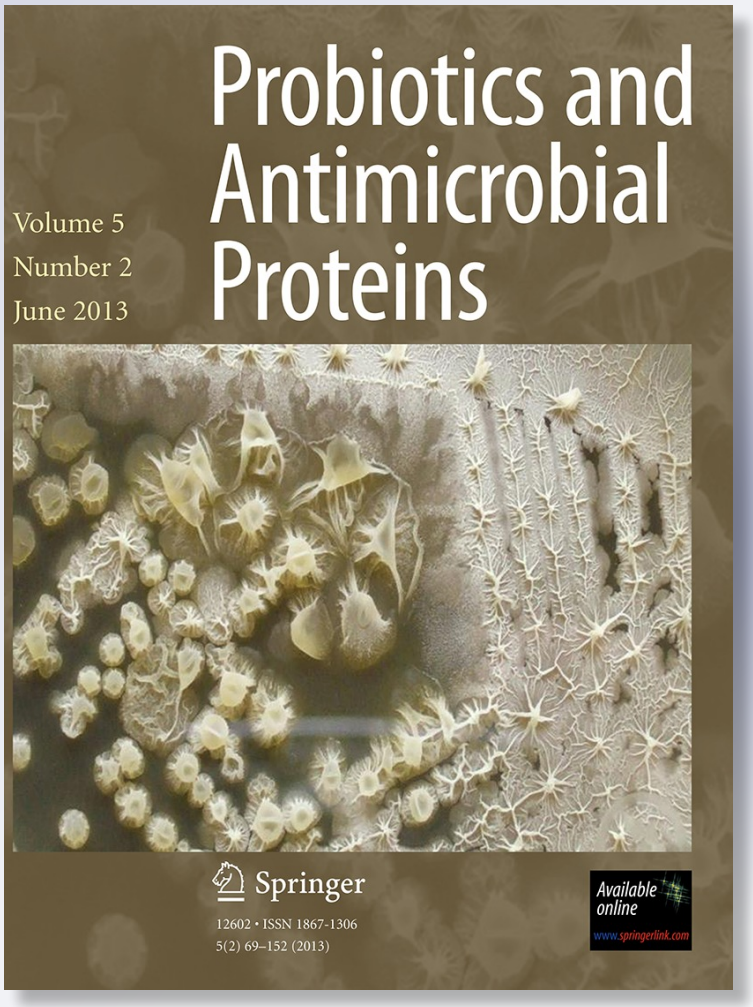


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# Partial Purification and Characterization of a Bacteriocin DT24 Produced by Probiotic Vaginal *Lactobacillus brevis* DT24 and Determination of its Anti-Uropathogenic *Escherichia coli* Potential

**Disha Trivedi · Prasant kumar Jena · Jignesh kumar Patel · Sriram Seshadri**

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**Abstract** The emergence of antibiotic resistance has increased the interest for finding new antimicrobials in the past decade. Probiotic Lactic acid bacteria producing antimicrobial proteins like bacteriocin can be excellent agents for development as novel therapeutic agents and complement to conventional antibiotic therapy. Uropathogenic *Escherichia coli*, most causative agent of Urinary tract infection, has developed resistance to various antibiotics. In the present investigation, antibacterial substance like bacteriocin (Bacteriocin DT24) produced by probiotic *Lactobacillus brevis* DT24 from vaginal sample of healthy Indian woman was partially purified and characterized. It was efficiently working against various pathogens, that is, Uropathogenic *E. coli*, *Enterococcus faecium*, *Enterococcus faecalis* and *Staphylococcus aureus*. The antimicrobial peptide was relatively heat resistant and also active over a broad range of pH 2–10. It has been partially purified by ammonium sulfate precipitation and gel filtration chromatography and checked on reverse-phase high-performance liquid chromatography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of bacteriocin DT24 was approximately 7-kDa protein. The peptide is inactivated by proteolytic enzymes, trypsin and lipase but not when treated with catalase, α-amylase and pepsin. It showed bacteriostatic mode of action against uropathogenic *E. coli*. Such characteristics indicate that this bacteriocin-producing probiotic may be a potential candidate for alternative agents to control urinary tract infections and other pathogens.

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**Keywords** *Lactobacillus brevis* · Uropathogenic *Escherichia coli* · Bacteriocin · Purification · Characterization · Mode of action

## Introduction

Urinary Tract Infection (UTI) remains one of the most common bacterial infections and second most common infectious disease in the community practice. Approximately about 150 million people were diagnosed with UTI each year [2]. Uropathogenic *Escherichia coli*, the primary etiologic agent for UTI, has developed resistance to most of the known traditionally used and third-generation antibiotics. The use of low-standard antibiotics further adds to the emerging drug resistance, which could turn to be one of the biggest factors for mortality in developing and underdeveloped countries [2]. Moreover, the antibiotics can be lethal to beneficial micro-organisms in the gastrointestinal tract (GIT) of human and animals, and they may also enter the food chain and accumulate in human body as undesirable chemical residues [50].

The vaginal microflora of healthy woman is dominated by lactobacilli species, at a level of $10^7$–$10^8$ CFU/g of vaginal fluid [47]. The antibacterial mechanisms of action of *Lactobacillus* appear to involve a lowering of the pH, the production of metabolites, such as hydrogen peroxide ($H_2O_2$) and lactic acid and of antibacterial molecules, including bacteriocins [46]. Ninety-six percent of *Lactobacillus* species found in healthy vaginal ecosystem produced $H_2O_2$ as one of its mode of action against pathogens [19]. $H_2O_2$-producing lactobacilli were used to prevent UTI [32], but recent studies have shown that cervicovaginal fluid, semen and spermicidal agent blocks the microbicidal activity of $H_2O_2$ produced by vaginal lactobacilli [40].

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Bacteriocin have drawn attention in recent years due to their potential therapeutic applications as an alternative to antibiotics in treating pathogenic bacteria, including multiple drug–resistant bacteria [11, 22]. Bacteriocin act toward species related to the producer with a very high potency and specificity. Various bacteriocins have been isolated from vaginal *Lactobacillus* spp. and have shown antibacterial potentials against different vaginal pathogens like *Gardnerella vaginalis*, *Candida albicans* and *Staphylococcus aureus* [4, 54].

Bacteriocins are a heterogeneous family of small, heat stable, ribosomally synthesized, extracellularly released bioactive peptides or proteins displaying antimicrobial activity against other bacteria and that are produced by many bacterial species, including probiotic strains [12]. They are generally low molecular weight proteins that enter into the target cells by binding to cell surface receptors [28]. The lactobacilli which produce bacteriocin are widely used in probiotic products for human and animal consumption to prevent pathogen growth in the GIT [5, 25, 37].

Principally, there are two different strategies for the administration of bacteriocins, that is, as a purified compound or in the form of bacteriocin-producing organism. Direct application of bacteriocins results in loss of its biological activity over time because of enzymatic degradation and interactions with proteins and lipids, which could be a major drawback [1, 21, 31, 48]. Hence, administration of probiotic organisms producing bacteriocin would be a better alternative.

In this study, *Lactobacillus brevis* DT24 producing bacteriocin was partially purified and characterized against uropathogenic *E. coli* and also checked for its probiotic potential for direct application as bacteriocin-producing probiotic for treatment of UTI.

In the present study, to the best of our knowledge, the partial purification and characterization of a bacteriocin DT24 produced by vaginal *L. brevis* DT24 showing antimicrobial activity against the indicator organism, uropathogenic *E. coli,* and other pathogenic Gram-negative and Gram-positive bacteria is reported for the first time.

## Materials and Methods

### Bacterial Strains, Growth Conditions and Media

*Lactobacillus brevis* DT24 (NCBI Accession No. JX163909) (Unpublished Data) isolated from vagina of healthy women was routinely propagated in MRS medium (Himedia, Mumbai). For bacteriocin production, the strain DT24 was grown in MRS medium. The indicator organism used in bacteriocin assay, uropathogenic *Escherichia coli* MTCC 729, was propagated in BHI broth. In addition, several Gram-positive and Gram-negative strains (Table 1), from various sources, were used in the determination of spectrum of activity. All cultures were raised at 30–37 °C in MRS or BHI broth as shown in Table 1. *Lactobacillus acidophilus* NCDC15 was used as standard strain for probiotic characterization was propogated in MRS medium (Himedia, Mumbai). All chemicals were obtained from Sigma Aldrich (USA) and all media components were purchased from Himedia (India). Antimicrobial activity was determined by the agar-spot-test method. Activity was expressed as arbitrary units (AU) per milliliter, with one AU defined as the reciprocal of the highest dilution showing a clear zone of inhibition [14].

### In Vitro Characterization of *L. brevis* DT24 Strain for Probiotic Characteristics

#### *Survival in Simulated Gastric Juice*

Analysis of tolerance to simulated gastric juice was conducted as described previously [36]. Simulated gastric fluid was prepared to assess passage through the upper GIT. The composition of simulated gastric juice was 1.28 g NaCl, 0.239 g KCl, 6.4 g $NaHCO_3$, 0.3 % bile salts (Central Drug House, Mumbai) and 0.1 % (w/v) pepsin (Himedia, Mumbai) per liter of distilled water, and the pH was 2.5.

Five milliliter overnight culture of grown *L. brevis* DT24 was collected by centrifugation and washed twice with 4 ml of Phosphate buffer saline (PBS; pH 7.0) and inoculated (at $10^{7-8}$ CFU/ml) in MRS broth with modifications for gastric juice tolerance medium then at times 0, 2, 4 and 6 h for determination of cell count by plating in triplicate onto MRS agar. The plates were incubated for 48 h. Then, the number of viable cells was determined by serial dilution and plate count method.

#### *Survival in Bile Salt*

*Lactobacillu brevis* DT24 grown overnight in MRS broth was adjusted to pH 6 and solution of bile salts (Oxoid) was added to a final concentration of 0.3 % (v/v).Samples were incubated for 6 h at 37 °C and aliquots were taken before adding bile salts and then at times 0, 2, 4 and 6 h for determination of cell count by plating in triplicate onto MRS agar. The plates were incubated for 48 h [39].

#### *Cell Surface Hydrophobicity Assay*

The surface hydrophobicity of *L. brevis* DT24 was determined by measuring the affinity of cells cultured overnight for toluene and xylene in a two phase system following the method of Ekmicki et al. [18]. Hydrophobicity was calculated from three replicates as the percent decrease in optical density of the original bacterial suspension due to

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**Table 1** Antagonism of bacteriocin DT24 produced by *Lactobacillus brevis* DT24 against various target strains

| Target strains | Collection | Growth medium | Growth temperature (°C) | Inhibition (mm) |
|---|---|---|---|---|
| *Escherichia coli* | MTCC 729 | BHI | 37 | 29 ± 0.03 |
| *Escherichia coli* | MTCC 443 | BHI | 37 | 26 ± 0.01 |
| *Lactobacillus helveticus PJ4* | Rat fecal isolate | MRS | 37 | 21 ± 0.32 |
| *Lactobacillus casei* | MTCC 1423 | MRS | 37 | 25 ± 0.16 |
| *Enterococcus faecium* | Vaginal isolate | MRS | 30 | 26 ± 0.27 |
| *Enterococcus faecalis* | Vaginal isolate | MRS | 30 | 23 ± 0.30 |
| *Staphylococcus aureus* | MTCC 737 | BHI | 37 | 21 ± 0.15 |
| *Salmonella* Typhimurium | MTCC 733 | BHI | 37 | 18 ± 0.19 |
| *Pseudomonas aeruginosa* | MTCC 1688 | BHI | 37 | 16 ± 0.21 |
| *Lactobacillus rhamnosus* | MTCC 1048 | MRS | 37 | 24 ± 0.60 |

*BHI* brain heart infusion agar, *MRS* De Man, Rogosa, Sharpe agar (Himedia)

Values are represented as mean ± SEM ($n = 3$)

cells partitioning into the hydrocarbon layer. The percentage cell surface hydrophobicity was calculated using the following equation:

$$\text{Hydrophobicity}\,\% = [(\text{OD600 before mixing} - \text{OD600 after mixing})/(\text{OD600 before mixing})] \times 100.$$

*Autoaggregation Assay*

Autoaggregation abilities were measured as described by Collado et al. [8], using the autoaggregation percentage. The percentage of autoaggregation was calculated as:

$$\text{Autoaggregation}\,(\%) = (A0 - At)/A0 \times 100,$$

where $A0$ represents the absorbance ($A600$ nm) at 0 h and $At$ represents the absorbance ($A600$ nm) at different time intervals (2, 4, 6, 12 and 24 h).

*Coaggregation Assay*

This experiment was performed according to the method described by Ekmicki et al. [18]. The *L. brevis* DT 24 was harvested by centrifugation at 14,000 rpm for 10 min, washed twice with sterile PBS (pH 7.0) and re-suspended in PBS. the number of bacteria ($10^7$–$10^8$ CFU/ml). This suspension (2 ml each) was mixed with 2 Absorbance (OD600) of bacterial suspensions was adjusted to 0.60 ± 0.02 in order to standardize ml of uropathogenic *E. coli* suspension for at least 10 s by a vortex mixer. After 4-h incubation, at 37 °C, the suspensions were measured at OD600 (Thermo-scientific, Spectronic). The coaggregation ability was expressed as follows:

$$\text{Coaggregation}\,\% = 100 \times [(\text{OD1} + \text{OD2}) - 2(\text{OD3})] / (\text{OD1} + \text{OD2}).$$

OD1 represents optical density of strain 1 (*L. brevis* DT24); OD2 represents optical density of strain 2 (Uropathogenic *E. coli*); OD3 represents optical density of mixed bacterial suspension containing *L. brevis* DT24 and uropathogenic *E. coli.*

Mode of Bacteriocin Action

*Growth of the Test Microorganisms in Presence of Bacteriocin*

BHI broth was inoculated with 1 % (v/v) uropathogenic *E. coli* at early exponential phase and then incubated at 37 °C. Ten-ml of filtered, sterilized cell-free supernatant was added to the cultures (90 ml) and changes in optical density (at 600 nm) were recorded every hour for 16 h. Control cells were treated with the inactive bacteriocin (treated for 30 min at 121 °C).

*Determination of the Reduction of Viable Cells of Target Microorganisms in the Presence of Bacteriocin*

Early stationary phase cultures of uropathogenic *E. coli* (16 h old) were harvested (10,000 rpm, 5 min, 4 °C), washed twice with sterile saline and re-suspended in 10-ml saline. Equal volumes of the cell suspensions and filter sterilized (0.20 μm, Axiva) bacteriocin DT24 were mixed. Viable cell numbers were determined before and after incubation for 1 h at 37 °C by plating onto suitable agar

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medium. Cell suspensions of uropathogenic *E. coli* and with no added bacteriocin served as controls.

### Molecular Mass Determination of bacteriocin DT24

A 24-h-old culture of *L. brevis* DT24 obtained in MRS broth at 37 °C was centrifuged (15 min, 10,000 rpm) and the pH adjusted to 6.0 with 1 N NaOH. To avoid proteolytic degradation of the bacteriocin, cell-free supernatants were treated for 10 min at 80 °C. Ammonium sulfate was slowly added to the cell-free supernatants to 60 % saturation and stirred for 4 h at 4 °C and centrifuged (10,000 rpm, 30 min, 4 °C). The precipitate was re-suspended in 10 ml of 25 mM ammonium acetate buffer (pH 6.5) and desalted by dialysis using a 1000-Da cut-off dialysis membrane (Sigma) against the same buffer. SDS–PAGE was used for further separation, as described by Schägger and VonJagow [45]. Low molecular weight markers with sizes ranging from 2.5 to 40.0 kDa (BioLit Protein marker, SRL, India) were used.

### Bacteriocin Purification

Bacteriocin was purified from a 1,000 ml culture of *L. brevis* DT24, grown in MRS broth as described above. Cells were removed by centrifugation at 14,000 rpm for 10 min at room temperature. Purification of bacteriocin was achieved by using a multistep protocol.

#### *Ammonium Sulfate Precipitation*

Culture supernatant was brought to 90 % saturation with solid ammonium sulfate, and after stirring overnight at 4 °C, the precipitate was collected by centrifugation (10,000 rpm, 10 min, 4 °C). The precipitate was dissolved in 60 ml sodium phosphate buffer (20 mM, pH 6.0), and the bacteriocin suspension was desalted by dialyzing through a 2-kDa cutoff dialysis membrane (Sigma) against the same buffer for 24 h. The dialyzed suspension was centrifuged at 14,000 rpm for 15 min at 4 °C. The supernatant was filtered through a 0.2-μm membrane and was checked for antimicrobial activity by agar well-diffusion assay by using uropathogenic *E. coli* as the indicator strain and the same was labeled as Fraction I [29].

#### *Gel Filtration Chromatography*

Fraction I was then loaded on Sephadex G-50 column and was eluted with 25 mM Sodium Phosphate buffer at a flow rate of 1 ml/min. The eluted fractions were checked for antimicrobial activity described earlier. The active fractions were pooled and named as Fraction II.

#### *Reverse-Phase High-Performance Liquid Chromatography*

To check the homogeneity of purified active fraction II, elute from the gel filtration chromatography was loaded in ACE-5, C-18-300 reverse-phase column (250 × 4.6 mm, ACE Capillary Column, Advance chromatography Technology, Scotland) using an HPLC system (Agilent, USA). The column was equilibrated with solvent A (HPLC grade water containing 0.1 % Trifluoroacetic acid (TFA)). The elution was performed using linear gradient from Solvent A to 100 % acetonitrile in 0.1 % TFA (Solvent B) for 60 min. The flow rate (0.3 ml/min) and temperature (60 °C) was maintained, and eluted analytes were monitored by an ultraviolet detector at 210 nm.

### Effect of pH, Temperature and Enzymes on the Activity of Bacteriocin

*Lactobacillus brevis* DT24 was grown in MRS medium for 20 h at 37 °C. The cells were harvested (10,000 rpm, 15 min, 4 °C) and the cell-free supernatant was incubated for 2 h in the presence of trypsin (Sigma), proteinase K (Sigma), pepsin (Sigma), lipase (Sigma) and α-amylase (Sigma) at final concentration of 1.0 mg/ml. Enzymes activity was terminated by boiling for 5 min and the residual activity was determined by using the well-diffusion method. An untreated sample was used as a control (100 %).

The effect of pH on the activity of bacteriocin DT24 was tested by adjusting cell-free supernatants from pH 2.0 to pH 10.0 (at increments of two pH units) with sterile 1 M NaOH or 1 M HCl for 1 h at 37 °C. After incubation, pH was set to pH 6.0. Antimicrobial activity was tested by well-diffusion method.

To determine the effects of temperature, purified antimicrobial samples were incubated independently at 30, 45, 60, 75 and 100 °C for 1 h. A non-heated sample was used as a control (100 %).

## Results and Discussion

For the common bacterium uropathogenic *E. coli* causing UTI [43, 44], antibiotic therapy remains the only standard and effective option, but excessive use leads to deleterious alterations of the normal host microbiota [17] and selection of resistant strains [13, 20, 26, 30, 38]. Hence, antimicrobial peptides like bacteriocin possessing specific antimicrobial activity against uropathogenic *E. coli* would be better alternative strategy for the treatment. Since direct application of bacteriocin may lead to loss of activity, application probiotic strain–producing bacteriocin would be better alternative. Hence, bacteriocin-producing probiotic

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lactobacilli preferably of the vaginal origin may be the better suited for UTI treatment. So, the aim of this study was to screen the antimicrobial protein–producing *Lactobacillus brevis* DT 24 from vagina of healthy women and to screen their bacteriocins as potential natural antibacterial agents for use against uropathogenic *E. coli* and few selected pathogens and characterization of probiotic potential of *L. brevis* DT24 like acid tolerance, bile tolerance, hydrophobicity, auto- and coaggregation.

## Spectrum of Activity

Bacteriocin DT24 presented a wide spectrum of activity, being inhibitory against uropathogenic *E. coli* and many Gram-positive, Gram-negative pathogens. The antibacterial activity of bacteriocin DT24 was not only evident against Gram-positive bacteria but also against Gram-negative bacteria (Table 1). Similar results were recorded for the cell-free supernatant and for the semi-purified bacteriocin. The bacteriocin DT24 showed high activity against uropathogenic *E. coli*, *S. aureus*, *E. faecalis* and *Enterococcus faecium* but showed low activity against *Pseudomonas aeruginosa* and Salmonella Typhimurium. Bacteriocin R1333 produced by *Lactobacillus sakei* R1333 isolated from smoked salmon and Enterocin LR/6, produced by *E. faecium* LR/6, were also active against Gram-negative bacteria [35, 57]. Earlier studies reported that bacteriocins of lactic acid bacteria are ineffective to inhibit Gram-negative bacteria since the outer membrane hinders the site for bacteriocin action [34]. However, a small number of bacteriocins produced by *Lactobacillus plantarum* have been reported to be active against Gram-negative bacteria. Bacteriocins ST26MS (2.8 kDa) and ST28MS (5.5 kDa) produced by *L. plantarum* ST26MS and ST28MS, respectively, can inhibit *Acinetobacter, Escherichia* and *Pseudomonas,* and antimicrobial activity against Gram-negative bacteria is also a significant characteristic that was earlier detected by several authors in other microorganisms including bacteriocins produced by other Lactobacilli [53].

## In Vitro Probiotic Characterization

### *Tolerance to Bile Acid and Artificial Gastric Acid*

*Lactobacillus brevis* DT24 was incubated for 0, 2, 4 and 6 h at 37 °C and assayed by viable cell counting. *L. brevis* DT24 showed lower survival rate than *Lactobacillus acidophillus* NCDC15 after incubation for 6 h (Table 2).This strain survived under the acidic condition (pH 2.5) for 6 h with cell number $10^{7.85}$ CFU/ml. The bile tolerance of *L. brevis* DT24 was also examined by its ability to grow 0.3 % bile (Oxgall), but *L. acidophillus* NCDC15 showed slightly higher tolerance with survival rate of 158. ± 0.11 % to 6 h compared to 154.03 ± 0.40 of *L. brevis* DT24 (Table 3).

To function efficiently as a probiotic strain, the probiotic bacteria must first survive transit through the stomach and exhibit their health-promoting effects as metabolically viable cells by reaching their action site [16]. The survival rate of *L. brevis* DT24 after 2 h of incubation was higher than several previously reported probiotic lactobacilli, including *Lactobacillus salivarius*, *Lactobacillus murinus*, *Lactobacillus pentosus* and *Lactobacillus delbrueckii* [7, 33]. From these results, it was suggested that *L. brevis* DT 24 can survive in a hostile environment (pH 2.5).

Bile plays a fundamental role in specific and non-specific defense mechanisms in the gut; the magnitude of its inhibitory effects is determined primarily by the concentrations of bile salts [49]. Bile tolerance is one of the prerequisite properties for probiotic bacteria to survive in the small intestine and to be functionally effective. *L. brevis* DT24 showed slightly lower bile tolerance than *L. acidophilus* NCDC 15.

### *Cell Surface Hydrophobicity, Autoaggregation and Coaggregation Assay*

The Cell surface hydrophobicity of *L. brevis* DT24 was examined in presence of toluene and xylene (Fig. 1). *L. brevis* DT24 showed higher hydrophobicity (39.00 ± 0.67 %) activity with toluene than that of *L. acidophillus* NCDC15 (33.22 ± 1.50 %) and with xylene (41.39 ± 0.51 %) than that of *L. acidophillus* NCDC15 (35.22 ± 0.51 %), respectively.

Autoaggregation ability of *L. brevis* DT24 was slightly lower (38.89 ± 0.10 %) compared to the standard strain *L. acidophillus* NCDC15 (38.89 ± 0.10 %) after incubated at room temperature for 12 h. This result indicates that the *L. brevis* DT24 possess potential ability to adhere to epithelial cells and mucosal surfaces (Fig. 2). *L. brevis* DT24 showed higher coaggregation (44.78 ± 0.35 %) efficiency than standard strains *L. acidophillus* NCDC15 (37.67 ± 1.00 %) with uropathogenic *E. coli* after 12 h incubation at room temperature (Fig. 3).

Physicochemical characteristics of the cell surface such as hydrophobicity may affect autoaggregation and coaggregation [9]. *L. brevis* DT24 showed higher hydrophobicity with toluene and xylene as compared to *L. acidophilus* NCDC15. The ability to adhere to epithelial cells and mucosal surfaces has been suggested to be an important property for probiotics. Cell adhesion is a multistep process involving contact of the bacterial cell membrane and interacting surfaces. Several workers have investigated the composition, structure and forces of interaction related to bacterial adhesion to intestinal epithelial cell [15, 41]. In most cases, aggregation ability is related to cell adherence properties [6]. A relationship between autoaggregation and adhesion ability has been

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**Table 2** Tolerence to artificial gastric acid

| Strains | Log CFU/ml | | | | % Viability |
|---|---|---|---|---|---|
| | 0 h | 2 h | 4 h | 6 h | |
| *L. brevis* DT24 | 7.68 ± 0.02 | 7.55 ± 0.03 | 7.21 ± 0.02 | 7.85 ± 0.02 | 102.26 ± 0.20 |
| *L. acidophilus* NCDC15 | 6.33 ± 0.02 | 6.21 ± 0.03 | 6.95 ± 0.04 | 7.48 ± 0.03 | 115 ± 0.28 |

Log mean counts of three trials (average ± SD) expressed as Log viable cell CFU/ml

Increase or % viability = $\log_{10}$ (final population) − $\log_{10}$ (initial population) expressed as increase or % survival. The values shown represent averages from triplicate experiments. Error represents the standard deviation. Data bearing different letter were significantly different ($p < 0.05$)

**Table 3** Tolerance to bile acid (0.3 % Oxgall)

| Stains | Log CFU/ml | | | | % Viability |
|---|---|---|---|---|---|
| | 0 h | 2 h | 4 h | 6 h | |
| *L. brevis* DT24 | 7.68 ± 0.02 | 7.64 ± 0.02 | 7.93 ± 0.04 | 8.22 ± 0.03 | 154.0 ± 0.40 |
| *L. acidophillus* NCDC15 | 5.33 ± 0.02 | 5.29 ± 0.02 | 5.47 ± 0.06 | 5.91 ± 0.02 | 158.0 ± 0.11 |

Log mean counts of three trials (average ± SD) expressed as Log viable cell CFU/ml

Increase or % viability = $\log_{10}$ (final population) − $\log_{10}$ (initial population) expressed as increase or % survival. The values shown represent averages from triplicate experiments. Error represents the SD. Data bearing different letter were significantly different ($p < 0.05$)

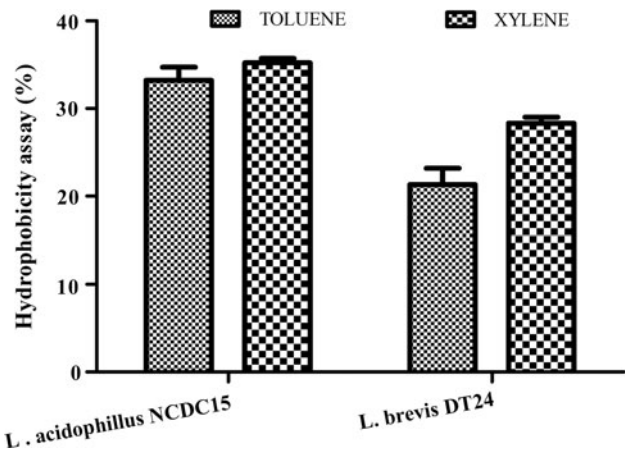


**Fig. 1** Cell surface hydrophobicity of *L. brevis* DT24 showed in percentage (%). The values shown represent averages from triplicate experiments

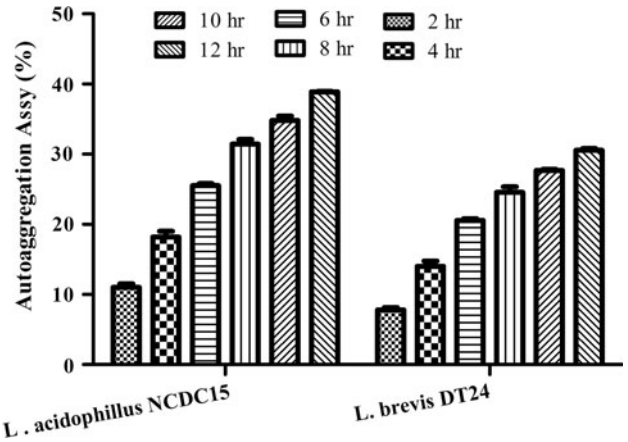


**Fig. 2** Autoaggregation ability of *L. brevis* DT24 strains in percentage (%). The values shown represent averages from triplicate experiments. *Error bars* represent the standard deviation

reported for some Bifidobacteria species [15]. In the present study, the isolates *L. brevis* DT 24 showed higher autoaggregation potential as compare to *L. acidophilus* NCDC 15.

## Mode of Action

Addition of cell-free supernatant of a 24-h-old culture of *L. brevis* DT24 to a 3-h-old culture of uropathogenic *E. coli* (early exponential phase) repressed cell growth of the indicator strains over 16 h (Fig. 4). Uropathogenic *E. coli* treated with bacteriocin DT24 (1,600 AU/ml) increased from OD600 0.142 to 0.457 over 7 h. The control uropathogenic *E. coli* (not treated with bacteriocin DT24) increased from OD600 0.142 to 1.094 over the same period (Fig. 4).

When stationary phase cells of uropathogenic *E. coli* ($10^7$–$10^8$ CFU/ml) were treated with the bacteriocin produced by *L. brevis* DT24 after 1-h contact time, low levels ($10^2$–$10^3$ CFU/ml) of uropathogenic *E. coli* cells were detected underlining the bactericidal mode of action of this bacteriocin. No significant changes in cell numbers of *E. coli* were recorded in the untreated (control) sample.

Earlier, similar results were obtained by bacteriocins HA-6111-2 and HA-5692-3 produced by *Pediococcus acidilactici* [3], Pediocin-like bacteriocin ST5Ha from *E. faecium* [55] and bacteriocin from *L. acidophilus* La-14 [56].

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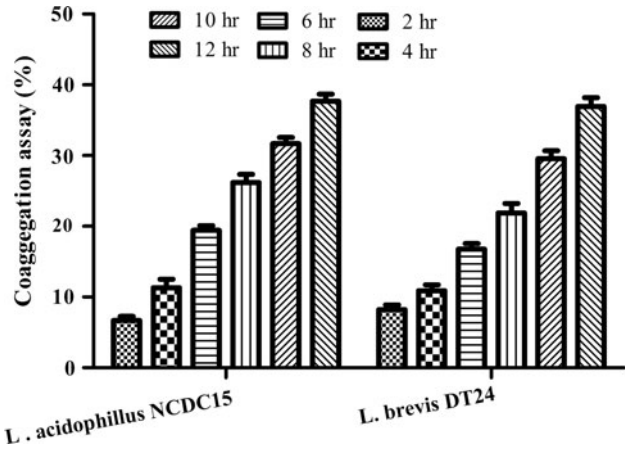


**Fig. 3** Coaggregation ability of *L. brevis* DT24 strain with pathogens showed in percentage. The values shown represent averages from triplicate experiments. *Error bars* represent the SD

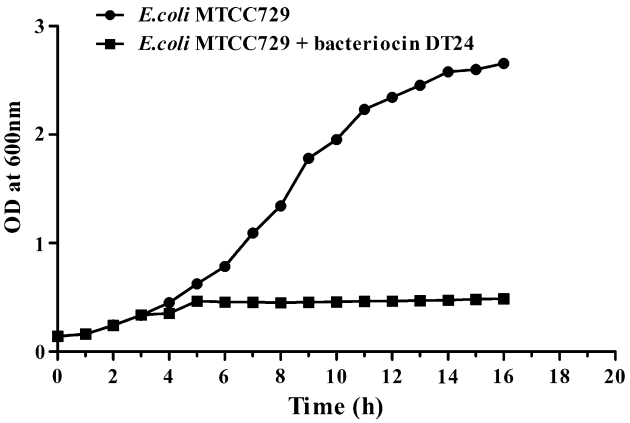


**Fig. 4** The effect of Bacteriocin DT24 on the growth of *E. coli* MTCC729. Growth of *E. coli* MTCC 729 in presence of Bacteriocin DT24 (*squares*) and in the absence of Bacteriocin DT24 (*circle*). The *arrow* indicates the time point at which the bacteriocin was added

## Molecular Mass Determination

SDS–PAGE was performed for the crude bacteriocin after ammonium sulfate precipitation (Fig. 5) and further by gel filtration chromatography. Due to larger protein mixture (Fig. 5a), the ammonium sulfate precipitate bacteriocin was further purified by gel filtration chromatography. According to tricine–SDS–PAGE, bacteriocin DT24 was estimated to be in the size range of around 7 kDa (Fig. 5b). This is within the size range of most bacteriocins reported for the genus *Lactobacillus* [36].

## Bacteriocin Purification

Bacteriocin DT24 was produced by *L. brevis* DT24 was purified from CFS to homogeneity. By ammonium sulfate precipitation, Sephadex G-50 gel filtration chromatography and the purity of bacteriocin DT24 was tested by RP-HPLC

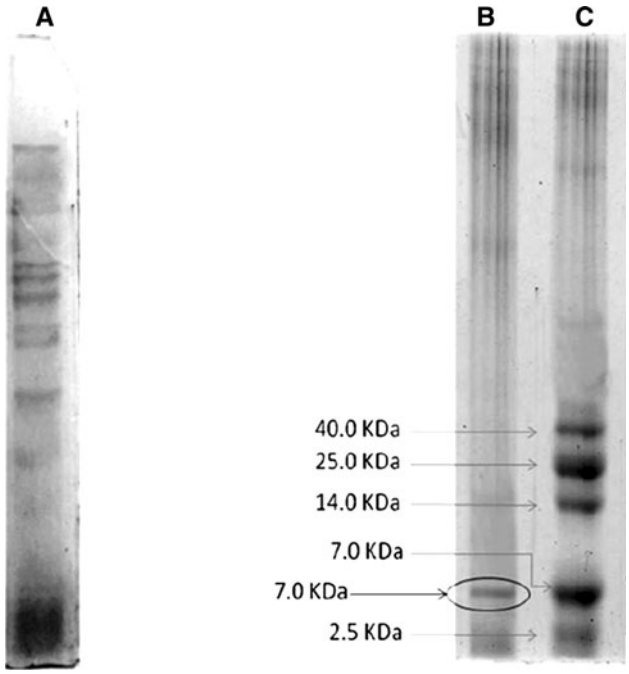


**Fig. 5** SDS–PAGE of bacteriocin DT24 (**a**) SDS–PAGE of protein mixture of ammonium sulfate precipitates (**b**) molecular mass marker (**c**) active fractions from gel filtration chromatography of Bacteriocin DT24 stained with Coomassie Blue R250

(ACE-5, C-18-300, 250 × 4.6 mm, ACE Capillary Column) chromatography. Cell-free supernatants from 24-h cultures in MRS broth at 37 °C were used for bacteriocin purification.

The activity against Uropathogenic *E. coli* presented by the proteins precipitated with ammonium sulfate and reconstituted in ammonium acetate buffer was similar to that presented by the fractions after gel filtration chromatography on Sephadex G-50 column. The antimicrobial activity of fraction I and fraction II was showing zone of inhibition of around 29 ± 0.31 mm, while the fraction after gel filtration chromatography showed around 31 ± 0.43 mm inhibition of zone. The HPLC analysis of crude bacteriocin after ammonium sulfate precipitation is shown in Fig. 6a. After gel filtration chromatography analysis (Fig. 6b), the active fraction II was re-injected in RP-HPLC. A partial purified active fraction was obtained at retention time of 38.74 min. Similar purification protocol was used by other researchers for purification of bacteriocins [23, 24, 35, 55].

## Effect of pH, Temperature and Enzymes on the Activity of Bacteriocin

The antimicrobial protein purified from *L. brevis* DT24 (bacteriocin DT24) was tested for their sensitivity to pH, temperature and enzymes; the results are summarized in Table 4. Bacteriocin DT24 remained stable after incubation for 1 h at pH values from 2.0 to pH 8.0, but its activity

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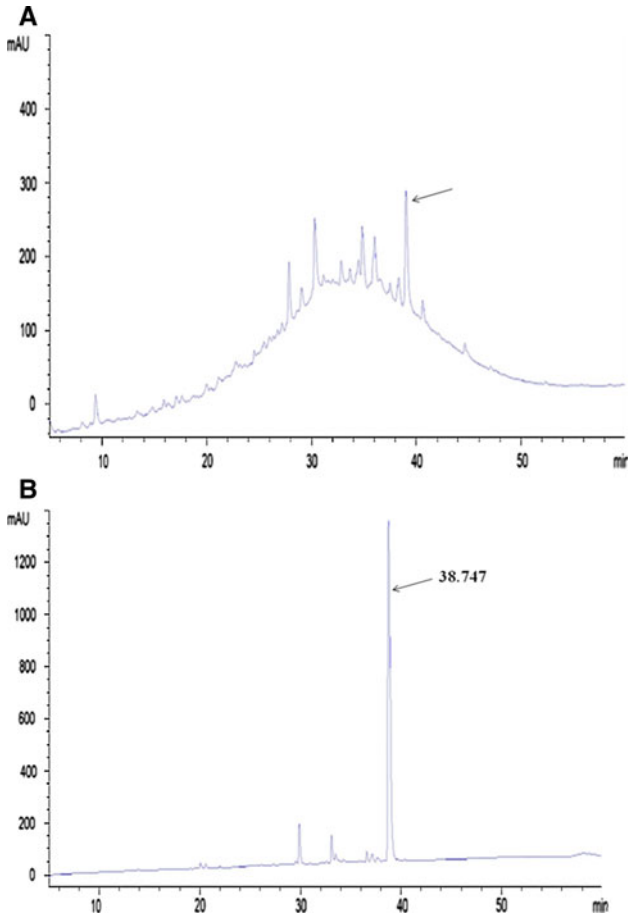


**Fig. 6** HPLC Chromatogram of primary separation after ammonium sulfate precipitation (**a**), and the active fraction again injected in RP-HPLC for protein purification peak (**b**) (retention time 38.74)

was reduced during pH 9.0–10.0 and highest activity was reached at pH 6.0. These results confirmed that the bacteriocin was resistant to acidic conditions. Bacteriocins were similar with regard to their sensitivity to inactivation by temperature. Like most of the known bacteriocins, they were mainly heat tolerant at pH 6.5 [51]. The bacteriocin DT24 showed greater thermal stability. It retained 96.19 % of its initial activity after 1-h incubation at 60 °C, whereas it retained only 73.39 % activity at 100 °C.

Results from enzyme inactivation studies established that antimicrobial activity was gone or unstable after treatment with all the proteolytic enzymes like proteinase K, trypsin and pepsin, whereas treatment with lipase, catalase, lysozyme and α-amylase did not change the activity of antimicrobial substance produced by *L. brevis* DT24, confirming its protein status. The sensitivity of the found substance to proteolytic enzymes is a proof of its proteinaceous nature, which allows considering it a bacteriocin. The other enzymes tested in our study (amylase and lipase) did not cause any inactivation. This could be explained with the lack of lipid or carbohydrate moiety in the

**Table 4** Effects of pH, temperature and enzymes on the activity of bacteriocin DT24

| Treatment | Residual antimicrobial activity (%) |
|---|---|
| pH | |
| 2 | 77.8 ± 0.43 |
| 4 | 80.12 ± 0.30 |
| 6 | 94.9 ± 0.12 |
| 8 | 61.2 ± 0.21 |
| 10 | 42.5 ± 0.13 |
| Temperature | |
| 30 | 100 |
| 45 | 100 |
| 60 | 96.19 ± 0.15 |
| 75 | 85.50 ± 0.34 |
| 100 | 73.39 ± 0.90 |
| Enzymes | |
| α-amylase | 100 |
| Pepsin | 89.04 ± 0.60 |
| Proteinase K | 0 |
| Trypsin | 0 |
| Lipase | 0 |
| Catalase | 93 ± 0.49 |

Values are represented as mean ± SEM ($n = 3$)

bacteriocin DT24. In addition, antimicrobial activity in this study was not due to hydrogen peroxide or acidity, as activity was not lost after treatment with catalase or peroxidase or adjustment of pH to 7.0. Similar results were reported by Todorov and Dicks [52], Corsetti et al. [10], Rattanachaikunsopon and Phumkhachorn [42], Hernandez et al. [27] and many other researchers.

## Conclusion

The antimicrobial activity of probiotic *Lactobacillus* can be due to many factors including low pH levels, competition for substrates and the production of substances with a bactericidal or bacteriostatic action, including bacteriocins. The antimicrobial components produced by probiotic strains may help to avoid pathogen colonization of mammalian vagina. These antimicrobial components may have extensive applications in clinical studies.

From the available datum, none of the bacteriocins isolated from probiotic and GRAS category lactobacilli are active against uropathogenic *E. coli.* This is the first report of characterizing the bacteriocin component of probiotic *L. brevis* DT 24 isolated from the vagina of healthy Indian women. The bacteriocin was most effectively inhibiting the growth of various pathogenic strains like, Uropathogenic *E. coli, E. faecalis, S. aureus* and *E. faecium.* The partially purified bacteriocin was not only biodegradable, but also

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stables in a wide range of pH values (2.0–10.0), heat resistant (30–100 °C) and size about 7 kDa. The bacteriocin DT24 producing strain *L. brevis* DT24 may have potential application in the prevention and treatment of UTIs and other gastro intestinal diseases. However, this needs to be confirmed in future in vivo experiments. In this study, we report the partial characterizations of the antimicrobial compounds bacteriocin DT24; however, in vivo efficacy of probiotic strain *L. brevis* DT24 and its purified bacteriocin DT24 and the identification and chemical characterizations of these compounds must be carried out to elucidate their complete structure and functioning.

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**Conflict of interest** The author(s) herewith certifies that there is no financial or commercial or proprietary interest in the products or companies described in the manuscript. There is no Conflict of Interest among the authors.

**Ethical Standard** All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation as per Institutional ethical committee, Nirma University (Ahmedabad). Protocol No. IEC/NU/I/IS/03 and with the Helsinki Declaration of 1975, as revised in 2008 (5). Informed consent was obtained from all patients for being included in the study. Additional informed consent was obtained from all patients for which identifying information is included in this article. This article does not contain any studies with animal subjects.

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