RESEARCH ARTICLE

Growth and antimicrobial activity of proteolytic probiotic
*Lactobacillus rhamnosus* C6 in soymilk and whey

Priyanka Kumari and Shilpa Vij

Received: 20 September 2014/ Accepted: 27 February 2015

**Abstract**  *Lactobacillus rhamnosus* is one of the most common bacteria in the gastro intestinal tract of healthy individuals. *L.rhamnosus* C6 (isolated from cheese) a proteolytic bacteria with proteolytic activity 509.12 μg serine/ml. *L.rhamnosus* C6 also showed the probiotic attributes as it survived at low pH (pH 1.0) and high bile concentration (up to 2%) having cell surface hydrophobicity for n-hexadecane (28.53 ± 0.37 %). The culture grown in broth also possessed antimicrobial activity against test pathogens (diameter of zone of inhibition ranging 17.76 to 23.77 mm) and showed good growth (9.19 log cfu/ml) in whey and also in soy milk (8.88 cfu/ml) after 48 h of incubation. *L.rhamnosus* C6 fermentate of whey and soy milk showed antimicrobial activity against *S. typhi* NCTC 6017, *S. aureus* MTCC 1144, *S. dysenteriae* NCDC 107, *L. monocytogenes* ATCC 15303, *B. cereus* ATCC 13061 and *E. coli* 0157:H7 ATCC 35150. So, the results of our study revealed that *L.rhamnosus* C6 is a good proteolytic as well as probiotic organism. It can grow well in cheese whey as well as soy milk and have antimicrobial activity in both.

**Keywords**: *Lactobacillus rhamnosus* C6, soymilk, whey, antimicrobial activity, probiotics

**Introduction**

*Lactobacillus rhamnosus* is one of the most common bacteria in the bowels of healthy individuals and it is the most extensively used as probiotic in clinical trials (Zocco et al., 2006). *L.rhamnosus* widely studied probiotic bacteria, has the ability to tolerate harsh condition of the gastro intestinal tract. *L.rhamnosus* GG has the properties beneficial for the intestinal tract. *L.rhamnosus* showed good probiotic as well as good proteolytic characteristics and survived under low pH conditions for 5 h and they tolerated well the bile acids (Fernandez et al., 2003). The survival of *L.rhamnosus* GG post 2 h incubation in MRS containing 3% bile salts had shown survival of 5.34 % (Gaudana et al., 2010). *L.rhamnosus* has proteolytic system ensures the growth in any medium. This system comprises proteinases located in the cell wall that allows the degradation of caseins into oligopeptides (Juille et al., 2003). The second part of the proteolytic system is the peptide transport system which allows the transit of oligopeptides released within the cell. The intracellular peptidases that hydrolyze oligo peptides into peptides or amino acids constitute the last part of this system. Nowadays, the concept of food having medicinal value has been reborn as ‘functional foods’ which gives health benefits. The gut is an obvious target for the development of functional foods, because it acts as an interface between the diet and all other body functions. Besides the nutritional values, ingestion of lactic acid bacteria (LAB) and their fermented foods have been suggested to confer a range of health benefits including immune system modulation, increased resistance to malignancy, and infectious illness (Soccol, et al., 2010). Soy (Glycine max) is considered as a potential source of proteins and good medium for growth of lactic acid bacteria and soy is used as substrate for functional foods. Fermentation of soy by lactic acid bacteria has the potential to (1) reduce the levels of some carbohydrates which can be responsible for gas production in the intestinal system, (2) increase free isoflavone levels (Wei et al., 2007) and (3) favor desirable changes in bacterial populations in the gastrointestinal tract (Bouhnik et al., 2004).
(Pinthong et al., 1980) have reported the reduction of raffinose and stachyose during fermentation of soymilk using pure or mixed culture of following bacteria: *Lactobacillus cellobiosis, L. planatarum, L. fermentum, L. delbrueckii, L. fermentum, L. pentosaceus* and *L. bulgaricus, Streptococcus thermophilus*. Soymilk fermentation with lactic acid bacteria is known to provide value addition by reducing the be any flavor and content of indigestible oligosaccharides and by enhancing the bioavailability of isoflavones, resulting in a nutritious probiotic food product (Telang et al., 2010). Whey is a by-product of the manufacture of cheese or casein and has several commercial uses. Whey is undoubtedly an excellent growth medium for various types of microorganisms because consists of primarily of proteins α-lactalbumin, β-lactoglobulin and glycomacropeptides (GMP) and lactose which is suitable medium for growth of *Lactobacilli*. Whey protein is recognized as a valuable food ingredient with important nutritional and functional properties is gaining acceptance as functional food ingredient. Whey proteins have a high content of sulfur-containing amino acids, which support antioxidant functions so, much attention is focused on whey proteins and whey protein hydrolysis. Hydrolyzed whey protein-based formulas are beneficial for infants intolerant to cow's milk protein. During hydrolysis proteins are broken down into peptides of different sizes and free essential amino acids (Lahl and Windstaff, 1989). Therefore, this study was carried out to investigate the proteolytic and probiotic activity of the *L. rhamnosus* C6 and its growth and antimicrobial activity in soy milk and whey during fermentation.

**Materials and Methods**

Whey was procured from Experimental Dairy, National Dairy Research Institute, Karnal. Whey was initially clarified through cheese cloth to remove casein particles and 6.0 pH was adjusted and autoclaved, and then stored at 4°C until required.

Soy milk was prepared by total of 1 kg of dry, mature, whole soybeans which were soaked in 3 L of distilled water at 25°C for 24 h. The soak water was then decanted, and the beans were washed then blanching for 30 min. and ground in 4 L of distilled water in a blender and the resulting suspension was filtered through three layers of cheese cloth, pH was adjusted to 6.5 and autoclaved for 15 min at 121°C, and then stored at 4°C until required.

Microbial cultures and their maintenance

*L. rhamnosus* C6 (KP844651) was isolated from cheese and identified by 16s RNA sequencing. *S. typhi* NCTC 6017 was procured from National Collection of Type Cultures, England. *S. aureus* MTCC 1144 collected from Microbial Type Culture Collection, Chandigarh, India. *S. dysenteriae* NCDC 107 collected from National Collection of Dairy Culture, National Dairy Research Institute, Karnal, India. *L. monocytogenes* ATCC 15303, *B. cereus* ATCC 13061, *E. coli* 0157:H7 ATCC 35150 was collected from American Type Culture Collection, USA. *L. rhamnosus* C6 was maintained and propagated by sub culturing in MRS broth @ 1% inoculum at 37°C for 24 h and then transferred into whey and soy milk. All the pathogens were maintained in YPD broth (dextrose 10.0 g, peptone 10 g, yeast extract 0.5 g, pH 7.0, distilled water 1000 ml) and stored in YPD agar slants (dextrose 10.0 g, peptone 10 g, yeast extract 0.5 g, agar powder 18g, pH 7.0, distilled water 1000ml). The morphology of *L. rhamnosus* C6 was determined by standard Gram staining procedure. Catalase test was performed by adding a drop of 3% H₂O₂ solution and added on to the culture and closely observed for the effervescence, indicating positive test.

Screening for proteolytic activity Proteolytic activity of *L. rhamnosus* C6 was measured by qualitative and quantitative method (Church et al., 1983).

Qualitative estimation of proteolytic activity on skim milk agar

*L. rhamnosus* C6 was inoculated in MRS broth for 15-16 hrs at 37°C after that centrifuged at 1500 g for 10 minutes. Supernatant was transferred in another tube and kept in boiling water bath for 1-2 min for inactivation of any viable cell. 10 ml of skim milk and 90 ml of nutrient agar were autoclaved separately to avoid coagulation and charring of milk and later mixed aseptically and poured in plate and allowed to harden. Wells were cut on the milk agar plates and 20 μl of cell free supernatant, MRS broth as negative control and trypsin as positive control were poured in to wells and incubated at 37°C for 24 h. Proteolytic activity was demonstrated by a clearing zone in the medium surrounding the wells.

Quantitative estimation of proteolytic activity by OPA (o-phthalaldehyde) method

Measurement of proteolytic activity by OPA method

Culture was activated and then 2.5 ml of the fully activated culture was added to 5 ml of 0.75% trichloroacetic acid and allowed to stand for 10 min, and the mixture was filtered using Whatman filter paper 42. Then 150 μl of permeate was added to 3 ml of OPA reagent which was kept on ice and the absorbance of the solution was measured spectrophotometrically at 340 nm after 2 min. at room temperature (20°C) (Church et al. 2003). The proteolytic activity was expressed as serine equivalents, according to the standard curve using serine in a concentration range of 25-250 μg/ml.

Growth of *L. rhamnosus* C6 in whey and soy milk

*L. rhamnosus* C6 culture was inoculated by adding @ 2% in whey and soy milk individually and grown at 37°C for 48 hrs
and sample were drawn at regular interval for analysis of samples. The growth was measured as total viable counts, pH decline and acidity production at regular time intervals. Inoculum was prepared by inoculating *L. rhamnosus* C6 @ 2% from MRS broth to the 10 ml of soymilk and whey and incubated at 37°C for 24 hrs and then culture was drawn from these samples and 2-3 times sub culturing were done for adaptation of culture in soymilk and whey and then culture was inoculated in whey and soy milk @ 2% and the pH, acidity and viable counts were determined. pH was measured by pH meter; acidity was measured by using 0.1N NaOH and phenolphthalein indicator. 10 ml whey and soy milk were collected after the addition of culture at 6 hrs intervals and few drops of phenolphthalein indicator was added and titrated with 0.1N NaOH till pink colour persisted and percentage of lactic acid was calculated as

\[
\% \text{ Lactic acid} = \frac{0.009 V \times 100}{W} = 0.9V
\]

Where, \( V \) = volume of 0.1N NaOH solution consumed during titration

\( W \) = weight or volume of sample

For viable counts, the appropriate dilutions of the samples were added into the plates and MRS agar was added. The plates were incubated at 37°C for 24 hrs and counts taken as log cfu/ml.

**Probiotic attributes of *L. rhamnosus* C6**

Indian Council of Medical Research (ICMR) along with the Department of Biotechnology (DBT) to formulate guidelines for regulation of probiotic in the country. These guidelines define a set of parameters required *In vitro* tests to screen potential probiotic strains (i) Resistance to gastric acidity, (ii) Bile acid resistance, (iii) Antimicrobial activity against potentially pathogenic bacteria (acid and bacteriocin production), (iv) Ability to reduce pathogen adhesion to surfaces, (v) Bile salt hydrolase activity and animal studies to establish safety and in vivo animal and human studies to establish efficacy. All the *in vitro* parameters were evaluated as per FAO/WHO 2002 and ICMR-DBT guidelines for *L. rhamnosus* C6. The probiotic strain can act as an adjuvant and stimulate the immune system against pathogenic microorganisms (Desai, 2008).

**Low pH tolerance of *L. rhamnosus* C6**

The acid tolerance of culture was studied in pH values prevalent in the stomach at 37°C. MRS broth of different pH 1.0, 2.0 and 3.0 were taken to simulate acidic conditions of gut. MRS broth with pH 6.5 served as a control. Activated culture was added @ 1% for 24 h in MRS broth and incubated at 37°C. Cell suspension containing about 109 cells/ml was added to each pH solution of 1.0, 2.0 and 3.0 and control and mixed and incubated at 37°C for 3 hrs. 1 ml of culture was taken from each pH solution immediately (0 h) and after 1, 2, 3 h and serial (10 fold) dilutions were prepared using 0.1% peptone water. Appropriate dilutions were pour plated in MRS agar and incubated at 37°C for 24-48 h and colony forming units (cfu/ml) were counted.

**Tolerance to high bile concentrations**

The method was adapted from that described by Pereira and Gibson (2002). The bile salt solutions were prepared by using HiMedia oxgall powder. 10 g dry powder dissolved in 90ml distilled water. From this solution, final concentrations of 1% and 2% were prepared in MRS broth. MRS broth without oxgall (pH 6.5) was used as control. 10 ml of each solution was transferred into sterile test tubes. 10⁹ cells/ml of cell suspensions was added to each solution, i.e., 1%, 2% and control and incubated at 37°C. 1 ml of culture was taken out from each tube immediately and 0, 1, 2 and 3 h time intervals and 10 fold dilutions were prepared in sterile 9 ml of 0.1% peptone water and pour plating was done on MRS agar and incubated at 37°C for 24-48 h and colony forming units (cfu/ml) were counted.

**Cell surface hydrophobicity**

Adherence of organisms to hydrocarbons is a measure of ability of the organisms to adhere to the epithelial cells in the gut. Cell surface hydrophobicity was determined according to the method described by Rosenberg *et al.*, (1980) using n-hexadecane. Activated bacterial cells were suspended in 10 ml of their desired broth @ 1% at 37°C for 18 h. After that, cell suspensions were centrifuged and the cell pellets were washed twice with phosphate urea magnesium (PUM) buffer. The washed pellets were resuspended in PUM buffer and the absorbance was adjusted to a value of approx. 0.7-0.9 OD at 610 nm. *L. rhamnosus* C6 cell suspension (3.0 ml) and n-hexadecane (1.0 ml) were mixed by vortexing and incubated at 37°C for 10 min for temperature equilibration. The mixture was again vortexed briefly and incubated at 37°C for 1 h for phase separations and the hydrocarbon layer was allowed to rise completely. After 1 h, aqueous phase was removed carefully with a Pasteur pipette and the absorbance was measured using spectrophotometer at 610nm. The decrease in the absorbance was taken as a measure of the cell surface hydrophobicity (H %) as

\[
H \% = \frac{A - A_0}{A} \times 100
\]
Antimicrobial activity of \textit{L. rhamnosus} C6

\textit{L. rhamnosus} C6 was screened for antimicrobial activity against various test organisms like \textit{S. typhi} NCTC 6017, \textit{S. aureus} MTCC 1144, \textit{S. dysenteriae} NCDC 107, \textit{L. monocytogenes} ATCC 15303, \textit{B. cereus} ATCC 13061 and \textit{E. coli} 0157:H7 ATCC 35150 using agar well diffusion. To check the antimicrobial activity, nutrient agar plates were made and after solidification overlaid with 7 ml of soft agar inoculated with 100 \( \mu \)l of overnight active culture of indicator strains. The soft agar was allowed to solidify. The plates were kept undisturbed for 2 hrs under refrigeration and after that incubated at 37\(^\circ\)C. After 24 hrs of incubation, a clear zone of inhibition was observed and measured in mm.

Results and Discussions

Proteolytic activity of \textit{L. rhamnosus} C6

Proteolysis results in the breakdown of large and complex proteins into the smaller and simple peptides due to the proteolytic activity of enzyme proteinase and peptidases especially from lactic acid bacteria. Pre-formed amino acids are an absolute requirement or a growth stimulant for all lactic acid bacteria.

Qualitative estimation of proteolytic activity of \textit{L. rhamnosus} C6

Qualitatively the proteolytic activity of the \textit{L. rhamnosus} C6 culture was determined on skim milk agar. MRS broth was used as negative control and trypsin as positive control. No zone of hydrolysis for MRS broth but \textit{L. rhamnosus} C6 and trypsin showed clear zone of hydrolysis (Table 1).

Quantitative estimation of proteolytic activity of \textit{L. rhamnosus} C6 by OPA (o-phthaldialdehyde) methods

The proteolytic activity of the \textit{L. rhamnosus} C6 was estimated by OPA (o-phthaldialdehyde) method after 12 hr of incubation time at 37\(^\circ\)C (Table 1). The proteolytic activity was measured as 509.12 \( \pm \) 0.63 (\( \mu \)g serine/mL). The free amino acids released during fermentation of milk were bound to OPA reagent and increased the absorbance at 340 nm. Hati (2012) also reported that \textit{L. rhamnosus} C6 was having proteolytic activity as 565.83 (\( \mu \)g serine/mL). Probiotic strain \textit{L. helveticus} M92 showed the proteolytic (Begunovic et al., 2013) and \textit{L. plantarum} NRRL B-4004 was the most proteolytic which complete \( \beta \)-casein hydrolysis after 215 h (Khalid et al., 1990). From the qualitative and quantitative results, \textit{L. rhamnosus} C6 was considered as highly proteolytic bacteria.

Growth of \textit{L. rhamnosus} C6 in whey and soy milk

The selected strain for this study has been recognized as highly proteolytic and fast growing in whey and soy milk. The culture performance was assessed as the ability of the strain to produce organic acids as primary metabolite, which was measured by decline in pH and increase in acidity as shown in Fig. 1 &2. The growth was also assessed by viable
cell counts. For appropriate culture performance in whey and soy milk, the culture requires highly developed proteolytic system capable of providing essential compounds for the culture growth. During the culture growth in whey and soymilk, this culture induced pH reduction and acid development in both the medium from 6.6 to 3.51 in soymilk and from 6.03 to 3.53 in whey after the incubation period of 48 h (Fig. 1 & 2). The best performance of the culture was observed in case of whey in which acidity increased to 0.98 from initial 0.62 after 48 h of incubation as compared to increased acidity in soymilk which was 0.83 after 48 h of incubation because whey contain more lactose to convert lactic acid than soymilk. However, after 24 h of incubation, the strain showed no variation in increase in acidity and decline in pH (Fig. 1 and 2). The rate of decline in pH is indicative of the culture activity and performance when it comes to the culture application in the industry.

*L. rhamnosus* C6 attained viable cell counts of desired level in both the growth media after incubation of 48 hrs at 37°C (Fig. 1 and 2). Viable cell counts were 4.13 log cfu/ml in soymilk and 4.22 log cfu/ml in whey at 0 h of incubation. After 24 h of incubation, the viable cell counts reached to 6.78 and 7.26 log cfu/ml in soymilk and whey, respectively. However, the cell counts continued to increase in both the media and attained maximum number of cells in soymilk and whey (8.88 and 9.16 log cfu/ml, respectively) at the end of fermentation. The data showed the consistent and gradual increase in cell number of *L. rhamnosus* C6 in whey and soymilk. However, decrease in pH during fermentation time did not affect the increase of growth level of the strain in whey and soymilk. This is a desirable property of the culture and could probably be due to the stability and ability of the culture to survive at various pH during fermentation. Wang et al., (2002) reported that little change in pH and acidity was found in soymilk fermented with single culture of *L. acidophilus*, although appreciable growth with a population increase of 2.65 log in time was observed. Such high viable counts was also reported

<table>
<thead>
<tr>
<th>Culture</th>
<th>Proteolytic activity (μg serine/ml)</th>
<th>Zones of proteolysis on skim milk</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. rhamnosus</em> C6</td>
<td>509.12 ±0.63</td>
<td></td>
</tr>
</tbody>
</table>

Table 1 Physico-chemical properties of aspartame and neotame

Fig 1 : Growth pattern of *L. rhamnosus* C6 in soy milk

Fig 2 : Growth pattern of *L. rhamnosus* C6 in whey
by Liu et al., (2011) who fermented soymilk with L.casei and observed 2.5 log increase after 14 h. Liu et al., (2006) reported 2.3 log increase in L.rhamnosus 6013 in soy cheese after 6h of fermentation. Hati (2012) studied the growth of L.rhamnosus C6 in soymilk for 12 hrs. Initial viable cell counts were 8.03 log cfu/ml in soymilk which reached 10.36 log cfu/ml, produced acidity of 0.70% and pH drop to 4.63 in 12 hrs of incubation.

Roy et al., (1986) reported that whey ultrafiltrate fermented by L.helveticus at 42ºC growth increases 4.7 to 6.3 log cfu/ml after 48 h of incubation.

Evaluation of probiotic attributes of L.rhamnosus C6

Tolerance to acidic pH

The gastric juice in stomach of pH 1.5-2.0 causes destruction of most of the microorganisms ingested (Charteris et al., 1998). In this sense, resistance to human gastric transit is an important selection criteria for probiotic organisms. The effect of different pH on the viability of L.rhamnosus C6 is presented in Table 2. Culture had survived pH 3.0 for 3 h, with viability of 6.40 ± 0.48 log counts. The initial log count (cfu/ml) of 8.12 decreased to 7.12 and 7.37 at pH 3 and 2 in 2 hrs respectively. There is a marginal decrease in log counts to low pH prevalent in stomach. Culture had also tolerated low acidic conditions of pH 2.0, with survival of 5.74 ± 0.92 log count after 3 h. However, the culture had shown minimum viability at pH 1.0 for 3 h with 0.63 ± 0.34 log count. At pH 6.5, L.rhamnosus C6 had shown increase in growth to log 9.78 ± 0.34 cfu/ml after 3 h. West et al., (1992) recorded fairly high acid tolerance of probiotic Lactobacillus cultures such as Lactobacillus GG, C1 and Y strains. A similar study was performed by Chou and Weimer., (1999) on L. acidophilus strains from the American Type Culture Collection. After 90 min of incubation at pH 3.5 all tested strains showed an optimal survival but no colonies were found in MRS agar incubated for 96 h at the same pH. L. rhamnosus strains from Parmigiano Reggiano cheese exhibited a good survival after 2 and 4 h of incubation at pH 3, but Chou and Weimer, (1999) reported that the long-term exposure to acidic environment causes a strong stress with a significant loss of survival.

Tolerance to high bile concentrations

Gastrointestinal systems have varying concentrations of bile, ranging between 0.5%-2.5% in the first hour of digestion and the levels may decrease further in subsequent hours. Tolerance to bile salts is considered to be a main prerequisite for growth, colonization and metabolic activity of bacteria in the gut. The bile salt tolerance pattern of culture is presented in Table 3. The culture had tolerated 1% bile with viability being 7.55±0.57 and 6.75 ± 0.95 log count after 2 and 3 h and at 2% bile concentration count decreased to 7.34±0.87 after 2hrs and further decreased to 5.98 ± 0.96 log counts after 3 hrs. However, at 2% bile concentration decrease in growth of L.rhamnosus C6 was more as compared to 1% bile. Only one log cycle reduction in counts were observed at 1% and 2% bile concentration in MRS broth after 2 hrs which may be more drastic conditions of bile concentration than present in stomach. L. rhamnosus strains isolated from Parmigiano Reggiano cheese showed good survival in presence of 1.0%, 1.5% and 2.0% bile salts (Succi et al. 2005)

Cell surface hydrophobicity

Adhesion to gut epithelial cells is an important property of

| Table 2  Acid tolerance of L.rhamnosus C6 |
|---------|-------------------------------|
| Time (h) | Viable cell counts (log cfu/ml) |
|          | pH 1.0 | pH 2.0 | pH 3.0 | pH 6.5 |
| 0        | 8.12±0.57 | 8.31±1.41 | 8.33±1.69 | 8.26±0.85 |
| 1        | 6.90±1.73 | 7.99±0.69 | 7.42±0.47 | 8.79±0.86 |
| 2        | 3.63±1.97 | 7.37±0.98 | 7.12±0.98 | 9.02±0.97 |
| 3        | 0.63±0.34 | 5.74±0.92 | 6.40±0.48 | 9.78±0.34 |

Values are mean ± SEM of three independent experiments

| Table 3  Bile tolerance of L.rhamnosus C6 |
|---------|-------------------------------|
| Time (h) | Viable cell counts (log cfu/ml) |
|          | Bile 0% | Bile 1% | Bile 2% |
| 0        | 8.32±0.77 | 8.68±0.76 | 8.41±0.93 |
| 1        | 9.04±0.96 | 8.43±0.93 | 8.60±0.42 |
| 2        | 9.32±0.85 | 7.55±0.57 | 7.34±0.87 |
| 3        | 9.81±0.57 | 6.75±0.95 | 5.98±0.96 |

Values are mean ± SEM of three independent experiments
a probiotic strain for temporary colonization of the GI tract and stimulation of beneficial effects. The cultures exhibiting higher cell surface hydrophobicity could be better performers in terms to adhere the intestinal epithelial cells (Perez et al., 1998). Culture was evaluated for its cell surface hydrophobicity towards hydrocarbon n-hexadecane (Table 4). L.rhamnosus C6 had shown 28.53 ± 0.37 % hydrophobicity for n-hexadecane. Higher hydrophobicity favors the colonization of mucosal surfaces and plays an important role in the adhesion of bacteria to epithelial cells. Kaushik et al., (2009) had observed hydrophobicity of L. acidophilus LA7 in the presence of n-hexadecane or xylene which ranges between 57-58% whereas hydrophobicity of some strains of L. acidophilus has been reported as low as 2-5% (Schillinger et al., 2005). The cell surface hydrophobicity varies from 2-95 % for different probiotic bacteria (Rijnaarts et al., 1993). The large differences in the cell surface hydrophobicity could be due to variation in the level of expression of cell surface proteins among strains of a species as well as due to environmental conditions which could affect the expression of surface proteins.

Antibiotic susceptibility test

The antibiotic susceptibility of probiotic culture can also influence their survival in the human gut, as the antibiotic therapy used to protect the gastrointestinal tract infections can also disbalance the normal microflora.

<table>
<thead>
<tr>
<th>Table 4</th>
<th>Cell surface hydrophobicity of L.rhamnosus C6 towards hydrocarbon n-Hexadecane</th>
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<tbody>
<tr>
<td>Hydrocarbons</td>
<td>% Hydrophobicity</td>
</tr>
<tr>
<td>n-hexadecane</td>
<td>28.53 ± 0.37</td>
</tr>
</tbody>
</table>

Values are mean ± SEM of three independent experiments

<table>
<thead>
<tr>
<th>Table 5</th>
<th>Antibiotic susceptibility of L.rhamnosus C6</th>
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</thead>
<tbody>
<tr>
<td>Antibiotics</td>
<td>Zone of inhibition</td>
</tr>
<tr>
<td>Amoxyclav (30 mcg)</td>
<td>23.67 ± 0.57</td>
</tr>
<tr>
<td>Ampicillin (10 mcg)</td>
<td>20.33 ± 0.51</td>
</tr>
<tr>
<td>Bacitracin (10 units)</td>
<td>13.67 ± 0.31</td>
</tr>
<tr>
<td>Chloramphenicol (30 mcg)</td>
<td>28.34 ± 0.55</td>
</tr>
<tr>
<td>Ciprofloxacin (5 mcg)</td>
<td>11.57 ± 0.36</td>
</tr>
<tr>
<td>Co-trimoxazole (1.25 mcg)</td>
<td>14.67 ± 0.57</td>
</tr>
<tr>
<td>Erythromycin (15 mcg)</td>
<td>22.33 ± 0.31</td>
</tr>
<tr>
<td>Gentamycin (10 mcg)</td>
<td>19.33 ± 0.57</td>
</tr>
<tr>
<td>Kanamycin (30 mcg)</td>
<td>00.00 ± 0.00</td>
</tr>
<tr>
<td>Ofloxacin (5 mcg)</td>
<td>15.67 ± 0.21</td>
</tr>
<tr>
<td>Penicillin-G (10 units)</td>
<td>28.67 ± 0.31</td>
</tr>
<tr>
<td>Vancomycin (30 mcg)</td>
<td>00.00 ± 0.00</td>
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</table>

Values are mean ± SEM of three independent experiments, pour plating in triplicate

This particular issue has generated a lot of interest for determining the antibiotic susceptibility or resistance of probiotics. The results of antibiogram of the culture investigated using disc diffusion assay for a total number of twelve clinically important antibiotics amoxyclav, ampicillin, chloramphenicol, gentamycin, penicillin-G, bacitracin, co-trimoxazole, erythromycin and ofloxacin according to Performance Standards for Antimicrobial Disk Susceptibility tests (CLSI, 2007) is shown in Table 5. L.rhamnosus C6 was susceptible to majority of antibiotics but very small zone of inhibition was observed in case of ciprofloxacin and no inhibitory zone was observed in case of vancomycin and kanamycin.

Davis et al., (2007) also recorded vancomycin resistance against all the Lactobacillus cultures. Vancomycin resistance is a widespread phenomenon among lactobacilli and resistance of heterofermentative and facultative heterofermentative lactobacilli to vancomycin is intrinsic, due to the presence of D-Ala-D-lactate in their peptidoglycan.

Antimicrobial activity of L. rhamnosus C6

The antimicrobial activity of probiotics is an important property for inhibition of the activities of pathogenic intestinal microflora. The inhibition of the growth of pathogens can be through the production of antimicrobial compounds like organic acid, hydrogen peroxide, bacteriocins etc. (Everett et al., 1996). Lactobacilli are the natural inhabitants of human gastrointestinal tract along with other microflora and these fermentative organisms produce organic acids (i.e. acetic and lactic acids), that tend to lower the intestinal pH inhibiting the multiplication of harmful microbes or pathogens. The agar well diffusion method was used to study antimicrobial activity of L.rhamnosus C6 against pathogenic organisms. Antimicrobial activity was determined by measuring the diameter of zone of inhibition (Table 6). The zones of inhibition of indicator
organisms tested were ranging between 17.76 ± 0.62 to 23.77 ± 0.35 mm in diameter. B. cereus was the most sensitive pathogen with diameter of zone of inhibition 23.77 ± 0.35 mm followed by Sh. dysenteriae (22.77 ± 0.57), L. monocytogenes (20.33 ± 0.48), S.aureus (20.33 ± 0.76), E. coli (19.53 ± 0.83), and S. typhi (17.76 ± 0.62) respectively. Singh et al. (2012) also reported that the L. casei subsp. casei NCDC17 and L.rhamnosus GG had antimicrobial activity against B. cereus, E. faecium, L. monocytogenes and S. aureus. Production of bacteriocin is one of the desired characteristic of probiotic organisms which show the antimicrobial activity against the closely related species. Antimicrobial activity of L.rhamnosus C6 may be due to the production of bacteriocin but here overall antimicrobial activity was determined. L.rhamnosus 68 showed the inhibitory activity against food spoilage bacteria and food-borne pathogens S.aureus (15±2.26 mm), E.coli (8.62±1.84 mm) and Bacillus sp. (11.25±2.25 mm) by the production of an inhibiting substance like-bacteriocin (Anas et al., 2008). The bacteriocin GP1 produced by L.rhamnosus GP1 isolated from Grape peel had wide spectrum of inhibitory activity against test strains of pathogenic and food spoilage micro organisms of B.brevis, B. pumilus, B. subtilis, E. coli, Pseudomonas aeruginosa, S.aureus, Vibrio harveyi, Acinetobacter sp. and Arthrobacter sp (Sarika et al., 2010).

Table 6  Antimicrobial activity of L.rhamnosus C6 grown in MRS broth by agar well diffusion assay

<table>
<thead>
<tr>
<th>Pathogens</th>
<th>Diameter of zone of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus</td>
<td>20.33±0.76</td>
</tr>
<tr>
<td>B. cereus</td>
<td>23.77±0.35</td>
</tr>
<tr>
<td>E. coli</td>
<td>19.53±0.83</td>
</tr>
<tr>
<td>L. monocytogenes</td>
<td>20.33±0.48</td>
</tr>
<tr>
<td>S. typhi</td>
<td>17.76±0.62</td>
</tr>
<tr>
<td>Sh. dysenteriae</td>
<td>22.77±0.57</td>
</tr>
</tbody>
</table>

Values are mean ± SEM of three independent experiments, pour plating in triplicate

Antimicrobial activity of whey and soymilk fermentates of L.rhamnosus C6

The culture performance of whey and soymilk fermentates of L.rhamnosus C6 was assessed as the antimicrobial activity against pathogens as shown in Fig. 3. The zones of inhibition of indicator organisms by soymilk fermentate were ranging between 13.00±1.53 mm to 17.33±1.53 mmin diameter (Fig.3). Sh. dysenteriae was the most sensitive pathogen with maximum zone of inhibition and L. monocytogenes was least sensitive towards soymilk fermentate. Similarly, the zones of inhibition of indicator organisms by whey fermentate were ranging between 11.33±0.57 to 12.66±0.58 mm in diameter (Fig.3) shown maximum zone of inhibition against Sh. dysenteriae and S.aureus and minimum against E. coli and
S. typhi, respectively. Whey fermented by *L. delbrueckii* subsp. *bulgaricus* SS1 and *Lactococcus lactis* subsp. *cremoris* FT4 exhibited antimicrobial activity against various Gram positive and Gram negative bacteria, e.g. *Escherichia*, *Helicobacter*, *Listeria*, *Salmonella* and *Staphylococcus* and also against yeasts and filamentous Fungi (Gobbetti et al. 2004).

**Conclusions**

From the foregoing sections, it can be concluded that the antimicrobial properties of *Lactobacillus rhamnosus* C6 is promising. *L.rhamnosus* C6 culture is having proteolytic activity of 509.12 μg serine/ml as well as probiotic properties on the basis of *in vitro* tests and its antimicrobial potency. *L.rhamnosus* C6 showed good growth in soy milk and whey. Both whey and soymilk fermentate of *L.rhamnosus* C6 showed the antimicrobial activity against pathogens but soymilk fermentate showed more antimicrobial activity than whey fermentate. The present study identified that *L.rhamnosus* C6 is a good probiotic culture which tolerate low pH, high bile concentration with good cell surface hydrophobicity, produce antimicrobial substances, which inhibited wide range of enteric pathogens both Gram positive and Gram negative. So, *L.rhamnosus* C6 can be used in the preparation of fermented functional foods with the production of health beneficial effects.

**References**


Khalid NM, Marth EH (1990) Proteolytic Activity by Strains of *Lactobacillus plantarum* and *Lactobacillus casei*.American Dairy Science Association, 73(11)-3068-3076

Lahj WI, Windstaff DA (1989) Spices and seasonings: hydrolysed proteins Proceedings of the 6th SIFST symposium on food ingredients - applications, status and safety, Singapore Institute of Food Science and Technology, Singapore 27-29 April, pp. 51-65


