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Research Article

In-situ control of spoilage microorganisms in presence of probiotic *Lactobacillus acidophilus* La 05 in Ricotta cheese

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Abstract

Ricotta is a high moisture and low acid soft spreadable cheese that is susceptible to spoilage due to the growth of various spoilage microbiotas. The current study indicated that the presence of probiotic organisms protected Ricotta cheese against natural spoilage organisms due to the presence of natural antimicrobial substances. Antimicrobial activity of cell-free supernatants of five probiotic organisms (*Lactobacillus acidophilus* La 05 NCDC 291, *Lactobacillus rhamnosus* NCDC 610, *Lactobacillus fermentum* NCDC 400, *Lactobacillus fermentum* NCDC 605, and *Lactobacillus reuteri* NCDC 22) was assessed against two indicator spoilage organisms (*Escherichia coli* NCDC 135 and *Staphylococcus aureus* NCDC 109). *Lactobacillus acidophilus* (NCDC 291) was selected based upon the maximum zone of inhibition (mm) against two indicator organisms, and used for the preparation of probiotic Ricotta cheese (PRC). The behavior of *Escherichia coli* and *Staphylococcus aureus* was assessed in the control and Ricotta cheese matrix during 12 days of storage at 7°C. In the presence of the probiotic organisms, a significant ($p<0.05$) reduction in the *E. coli* (20%) and *S. aureus* (23.4%) count was observed at the end of 12 days of storage with an inhibition rate of 9.47% and 11.62% against *Escherichia coli* and *Staphylococcus aureus*, respectively.

Key words: Antimicrobial activity, Probiotic, Ricotta cheese, Safety, Spoilage bacteria

Highlights

- Antimicrobial activity of five probiotic organisms was assessed against two spoilage organisms.
- Based upon maximum zone of inhibition (mm) *L. acidophilus* (NCDC 291) was selected.
- Probiotic Ricotta cheese (PRC) was prepared from buffalo milk using NCDC 291.
- The behaviour of *E. coli* and *S. aureus* was assessed in control and PRC during refrigerated storage.
- *E. coli* (20%) and *S. aureus* (23.4%) count reduced in PRC at the end of 12 days storage.

INTRODUCTION

Processing operations leading to conversion of raw materials into food products often generate several by-products. Whey is the largest by-product of the dairy industry in terms of volume and milk solids as it contains about 50% of milk solids. It is a source of huge nutrients including proteins, lactose, minerals, water soluble vitamins and residual lipids. Whey protein is considered second best protein in quality, having a biological value (BV), protein efficiency ratio (PER) and net protein utilization (NPU) of 104, 3.6 and 92, respectively as against corresponding respective values of 100, 3.8 and 94 for egg protein (Renner, 1990). The presence of essential amino acids in high amounts such as lysine, methionine and cystine (Swaisgood, 1995) make it nutritionally superior and beneficial for human health. The biological active components of whey can provide various health beneficial effects like enhancement of immunity, anti-oxidative, antihypertensive, antitumor, antiviral and antibacterial effects, etc. In spite of all these nutritional and functional properties, whey is usually disposed of, even in most organized dairy plants leading to huge loss of nutrients. Further, owing to high BOD value, whey disposal may cause severe environmental pollution and a real concern for dairy industry.

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Approximately 8.7 kg of whey is produced per kg of cheese manufactured. Sweet and acid whey, by-products of cheese and paneer industry, are extremely potent pollutants, containing about 35,000 mg/L biochemical oxygen demand (BOD) and 68,000 mg/L chemical oxygen demand (COD). Therefore, complete utilization of whey in food system has been under active consideration globally.

Most popular and oldest variety of whey cheese is Ricotta. It may be considered as a protein co-precipitate with high moisture content (Borba *et al*., 2014). Ricotta is a mellow, unripened cheese made from small ruminant's milk which restricts its availability mainly due to high price, low availability and output of small ruminant's milk. India has 56% of the world's buffalo population and 49% of milk produced in India is buffalo milk. As the mozzarella cheese industry is growing in India, leading to production of increased volume of whey, an interest is being generated in the production of buffalo milk Ricotta cheese.

When given in proper doses, probiotic organisms may support the host's health (Hill *et al*., 2014). Probiotic organisms modulate the immune system, decrease cholesterol, prevent cancer, improve lactose intolerance, and reduce diarrhoea, constipation and UTIs, etc. (Hill *et al*., 2014). A count of 10^6 CFU/g to 10^9 CFU/g of probiotic bacteria is considered good for providing health benefits (Ganguly *et al*., 2019). Probiotics can be bacteria, molds or yeasts, however, Lactobacilli and Bifidobacteria are the most prevalent probiotic organisms (Alard *et al*., 2018).

Probiotic organisms are recognised for their antibacterial action against spoilage and pathogenic micro-organisms apart from their health beneficial effects (Madureira *et al*., 2011). This is due to the formation of organic acids, bacteriocins, hydrogen peroxide, and other metabolites (Gomes and Malcata, 1999).

Ricotta may be a better delivery medium for probiotic microbes than other products as it contains high moisture, high pH, low salt content, and low-oxygen level, which may preserve probiotic microbes throughout storage and gastrointestinal transit. Probiotics may increase the sensory quality, shelf life, and functional characteristics of Ricotta cheese. Fresh Ricotta has a short shelf life and support growth of yeast, mould, and other pathogens and is a favourable substrate for microbiological contamination owing to its high nutrition and water content, which restricts its shelf life to 5-10 days. Yeast, mould, *E coli*, *Staphylococcus aureus*, and *Psuedomonas* spp. are common spoilage flora of Ricotta (Madureira *et al*., 2011). Ricotta is a rich source of nutrients in readily available form (e.g., lactose or peptides), high in pH and moisture content which supports the contaminant microorganisms to grow easily. The common spoilage flora of whey cheeses are *Staphylococcus*, *Pseudomonas*, *and Escherichia coli* which had been documented in vary high viable numbers in Ricotta and other whey chesses during storage (Govaris *et al*., 2001; Normanno *et al*., 2005). If such organisms are pathogenic, they can cause illness after ingestion (Madureira *et al*., 2011). High microbial contamination in Ricotta cheese can be managed by adding bacitracin-producing probiotic organisms, which may improve quality, safety, and shelf life (Moura *et al*., 2013). The current study aimed at evaluation of the impact of probiotic organism *L. acidophilus* LA 05 on shelf-life and safety against spoilage organisms in Ricotta cheese.

MATERIALS AND METHODS

Materials: Fresh buffalo milk with ~7.5% fat and 3.7% protein and 9.5% SNF, was procured from the Experimental Dairy of the National Dairy Research Institute, Karnal, India. Salt and citric acid were bought from standard manufacturers. Polystyrene tubs (200 g) with lids were obtained from the local market of Karnal, Haryana, India. Chemicals used during the study were purchased from Sigma Aldrich, USA. The probiotic bacteria namely, *L. acidophilus* La-05 (NCDC-291), *L. rhamnosus* (NCDC-610), *L. fermentum* (NCDC-400),

L. fermentum (NCDC-605), and *L. reuteri* (NCDC-22) were collected from the National Collection of Dairy Cultures (NCDC), NDRI, Karnal, India. All microbiological media were purchased from Hi-Media, Mumbai, India.

Collection and maintenance of probiotic organisms: The cultures of freeze-dried form were activated in MRS broth at 37°C for 24h. The organisms were grown subsequently sub-culturing twice before they were used for preparing the 16 h old inocula (count ~ 8 log CFU/mL) used in further experiments and product preparation. Stock cultures of Lactobacilli were maintained at -20°C in MRS containing 20% glycerol.

Collection of spoilage organism: *E. coli* (NCDC-135) and *Staphylococcus aureus* (NCDC-109) were obtained from the National Collection of Dairy Cultures (NCDC), NDRI, Karnal, India. Spoilage organisms were grown in Brain Heart Infusion broth.

Screening antimicrobial activity: Probiotic organisms were assessed for their antimicrobial activity (Jacobsen *et al*., 1999). For this MRS agar plates were overlaid with 7 mL of soft MRS agar inoculated with 50 µL of spoilage organisms which was overnight active culture. About five wells were made in agar using borer and filled with 100 µL cell-free supernatant of 24 h old probiotic cultures obtained by centrifugation of the culture at 8000 rpm for 15 min. The zone of inhibition (diameter) expanding nearby the well was determined and a clear zone of one mm or more was taken as a positive inhibition.

Preparation of probiotic buffalo milk Ricotta cheese (PRC): Probiotic Ricotta cheese was prepared following by the protocol as given by Bhagwat *et al.* (2020a). Mozzarella cheese whey was heat-treated to 70°C for 5 minutes. Pasteurized buffalo milk having 1% fat was admixed with whey in the ratio of 80:20 (whey: buffalo milk) followed by application of heat treatment to 90°C for 15 minutes and cooling to a coagulation temperature of 75°C. Coagulation was done using citric acid (1%) to obtain a pH of 5.4. Based on a maximum zone of inhibition against two spoilage organisms, one probiotic organism was selected and grown thrice in MRS medium at 37°C for 18 h. Cell pellets of the probiotic organism were obtained by centrifugation at 8000 rpm for 15 min at 4°C and washing in sterile saline solution (0.85% NaCl). The cell count was adjusted (~8 log CFU/mL) by measuring the optical density using a spectrophotometer (SHIMADZU, UV 1800) at a 625 nm (OD_{625}) wave length. Probiotic cells and salt (1% w/w) were incorporated into Ricotta cheese by homogenous mixing with a Hobart mixer. Probiotic Ricotta was packed in 200 g polystyrene tubs and kept for storage at 7°C. Moisture, fat (gravimetrically), and ash content of probiotic Ricotta cheese were determined as per the method described in IS: SP: 18 (Part XI- 1981). The total protein content (Micro-Kjeldahl method) and titratable acidity of cheese were measured as per methods suggested in AOAC (2005). The lactose content of cheese was calculated by difference. The water activity of Ricotta cheese was measured by using Aqua Lab aw meter (Decagon Devices Inc., Washington, USA).

Viable count of probiotic organism: Viable count of L. *acidophilus* La-05 was conducted using process given by Meira *et al.* (2015) for whey cheese. About 25 g of cheese sample was mixed aseptically in 225 mL saline (0.85% NaCl) and the mixture was diluted serially (10^1-10^8), which was used pour plating method and the enumeration of probiotic organism was done using salicin (0.5%) modified MRS medium, incubated at 37ºC for 48 h and probiotic count was expressed as log CFU per gm of cheese.

Inhibitory effect of the probiotic organism against spoilage organisms in Ricotta matrix: Spoilage and probiotic strains were serially diluted (10^1 to 10^8) and the optical density was

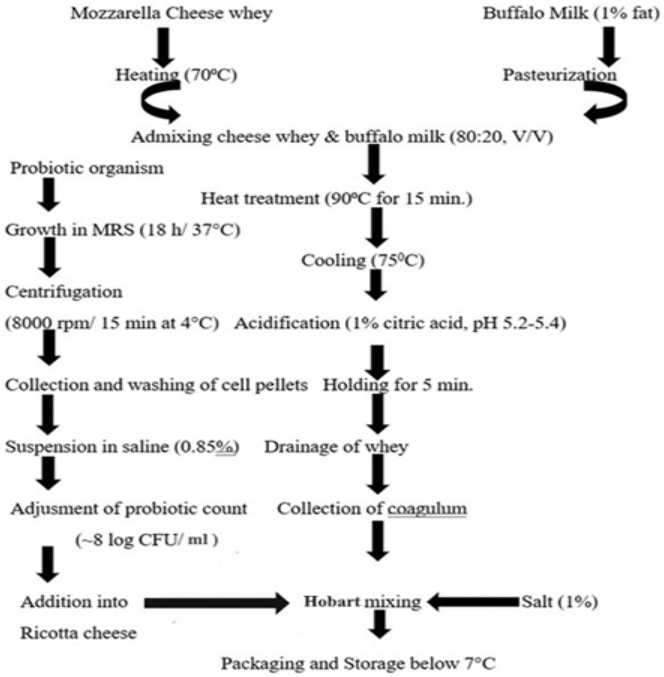


Fig. 1. Flow diagram of probiotic Ricotta cheese

measured for each dilution at a wavelength of 625 nm (OD_{625}), keeping saline solution blank, and OD was measured using a spectrophotometer (SHIMADZU, UV 1800). 1 mL of each dilution was plated on MRS agar and incubated for 48 h at 37°C, for enumeration of probiotic *L. acidophilus* La 05 and CFU/mL of strain was estimated in each OD_{625} reading. The 0.8 and 0.1 OD_{625} values were used to get the desired levels of *L. acidophilus* (8 log CFU/ mL) and spoilage bacteria (*E. coli* and *S. aureus*; ~5.5 log CFU/mL), respectively. Inhibitory effect of the probiotic organism against spoilage organisms in Ricotta matrix was conducted as per the protocol of Prezzi *et al.* (2020). Cheese of 25 g was divided and kept into sterile glass jars (250 mL) for further study. Control cheese contained cheese with spoilage bacteria alone and the experimental cheese contained probiotic bacteria with spoilage organism. Inoculum of 1 mL of each spoilage and probiotic bacteria was added to each 25 g of cheese providing a count of approximately 5.5 log CFU/g and 8 log CFU/ g, respectively for spoilage and probiotic bacteria. Ricotta cheese was inoculated with spoilage organism at 10^5 to 10^6 CFU/g which indicates a medium level of contamination (Coelho *et al.*, 2014). The inoculum of spoilage bacteria was fixed considering the studies of the real level of contamination as reported in fresh cheese samples (Aragon-Alegro *et al.*, 2007; Meneses *et al.*, 2012). Inoculated cheese samples were mixed in a Hobart mixer (Kenwood, UK) for 5 min followed by storage at 7°C for 12 days. Each sample was studied for a viable cell count of contaminants at 0, 6, and 12 days of interval of refrigerated storage using the pour plate technique. Violet Red Bile Agar (VRBA) and Baird Parker Agar (BPA) with egg yolk tellurite emulsion were used for enumeration of *E. coli* and *S. aureus*, respectively. All agar plates were incubated for 24-48 h at 37°C (American Public Health Association, 1992). All analyses were performed in triplicate and the results were expressed in log CFU per gram. For each storage period, the degree of inhibition (inhibition rate) was calculated using the below-given formula:

$$\text{Inhibition rate} = \frac{N_o - N_i}{N_o} \times 100 \quad \text{...Eq. (1)}$$

Where, N_0 is the number of viable cells of the contaminant (log CFU per gram) in the control sample (without probiotic) and N_i is the number of viable cells of the contaminant (log CFU per gram) in the presence of a probiotic bacteria at a given inoculum level.

Statistical analysis: The experimental results were analyzed using and SPSS 16.0 software. The significance of the difference between two or more treatments was tested by Student's t-test and analysis of variance (ANOVA) with Tukey's post hoc test at a 5% level of significance. The graphical representation of data was done by using Prism Graph-pad version 8.1.1.

RESULTS

Screening of probiotic strain: The antimicrobial activity of probiotics is one of the significant properties for inhibiting pathogenic microflora and making the host safer against infections. Similarly, inhibition of the growth of spoilage organisms can be controlled by the probiotic organism, which may enhance the shelf life of the product. Production of antimicrobial metabolites (organic acids, bacteriocins, hydrogen peroxides, etc.) and competitive exclusion among others could be considered as the major mechanisms for the antimicrobial activity of probiotic organisms (Musikasang *et al.*, 2009). The agar well diffusion method was used to study the antimicrobial activity against two spoilage-causing organisms (*E. coli* and *S. aureus*) and the zone of inhibition was measured. The zone of inhibition (mm) for probiotic organisms against spoilage organisms has been presented in Fig. 2A and Fig. 2B. The zone of inhibition against *E. coli* ranged from 9.06 mm to 12.90 mm.

Probiotic organism *L. acidophilus* La-5 (NCDC 291) showed a maximum zone of inhibition (14.17±0.15 mm), followed by *L. rhamnosus (*NCDC-610) (12.90±0.10 mm), *L. fermentum* (NCDC 605) (9.01±0.07 mm), *L. reuteri* (NCDC 400) (9.06±0.15 mm), and *L. fermentum* (NCDC 400) (7.6±0.12 mm). The zone of inhibition for *S. aureus* ranged from 10.17 mm to 13.01 mm. Maximum zone of inhibition was observed for *L. acidophilus* La-05 (NCDC 291) (14.22±0.10 mm) and minimum for *L. reuteri (*NCDC 400) (9.19±0.04 mm), as represented in Fig. 3.

Based on the maximum zone of inhibition (Table 1) from the antimicrobial activity against

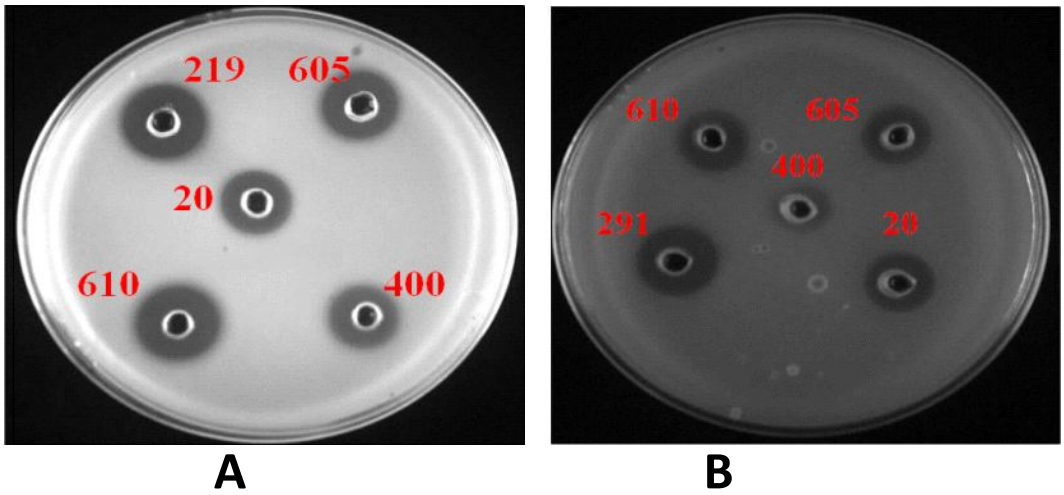
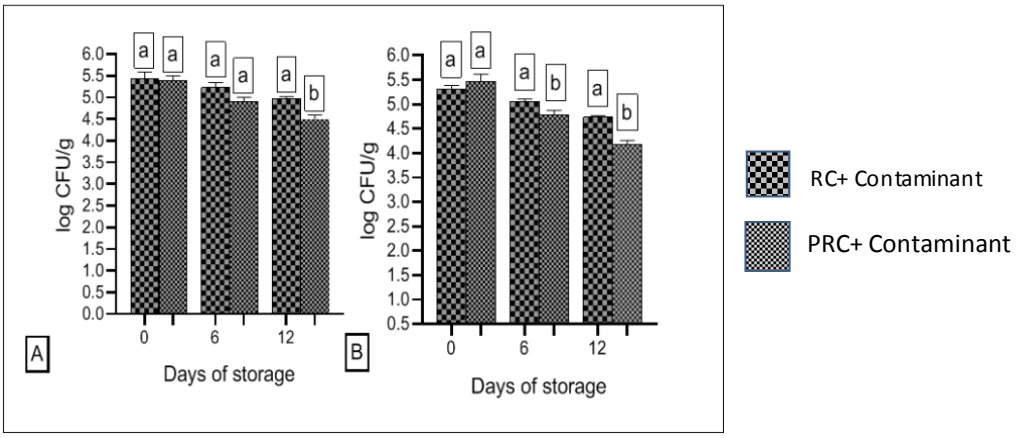


Fig. 2. Zone of inhibition of probiotic organisms against various spoilage organisms; A: *S. aureus*; B: *E. coli*



[A: Behaviour of *E. coli* (NCDC 135); B: Behaviour of *S. aureus* (NCDC 109). Values with different superscripted letters in the graph differs significantly within same group ($p<0.05$)]

Fig. 4. Behaviour of spoilage organism in control and probiotic Ricotta cheese during storage

Table 1. Composition of control and probiotic Ricotta cheese

Compositional parameters (% w/w)	Control Ricotta cheese	Probiotic Ricotta cheese
Total solids	24.86±0.13[a]	25.03±0.09[a]
Fat	6.67±0.03[a]	6.41±0.11[a]
Protein	11.67±0.18[a]	11.88±0.13[a]
Lactose	4.54±0.24[a]	4.88±0.19[a]
Ash	1.98±0.01[a]	1.86±0.03[a]
Water activity	0.99±0.001[a]	0.99±0.001[a]
Titratable acidity (% L.A.)	0.145±0.01[a]	0.145±0.01[a]
Probiotic count (log CFU/g)	-	7.8±0.2

Mean ± SD, n=3; values with same superscripted letters differ significantly ($p<0.05$)

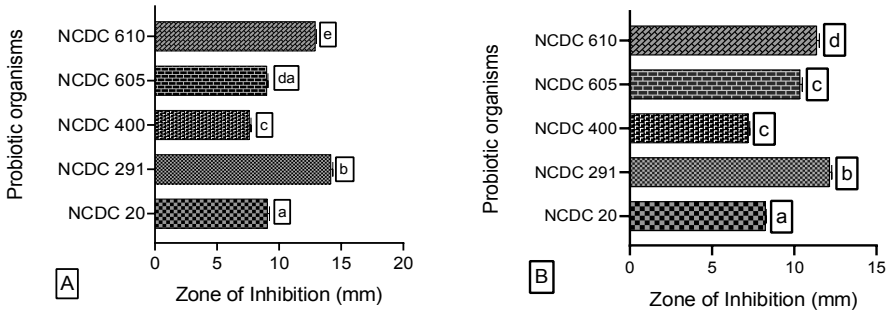
all spoilage organisms by agar well assay, *L. acidophilus* La 05 (NCDC 291) was selected for further studies.

Preparation and analysis of the composition of probiotic Ricotta cheese: Probiotic Ricotta was prepared by incorporation of selected probiotic *L. acidophilus* La 05 (NCDC 291) as represented in Fig. 1. The proximate composition of Ricotta and probiotic Ricotta cheese was similar. The total solids content of RC and PRC were 24.86% and 25.03%, respectively, which were statistically non-significant ($p>0.05$). The respective values for fat, protein, lactose and ash were 6.67%, 11.67%, 4.54% and 1.98% for RC, and 6.41%, 11.88%, 4.88%, and 1.86% for PRC, all the compositional parameters were statistically non-significant ($p>0.05$) for both the samples. The water activity value for both the samples was 0.99, while titratable acidity was 0.145%. The observed probiotic count in PRC was 7.8±0.2 log CFU/g of product. Probiotic incorporation did not modify the composition of Ricotta cheese, similarly, was reported by Meira *et al.* (2015). The product was acceptable to the panellists as revealed by the high overall acceptability scores (9.0) (Bhagwat *et al.*, 2020a); further probiotic organisms showed better survival in the Ricotta matrix as compared to the MRS medium during experimental digestion process due to buffering capacity of

milk protein which protected organism (Bhagwat *et al*., 2021).

Inhibitory effect of *L. acidophilus* La 05 in Ricotta matrix against spoilage organisms: The behaviour of spoilage organisms in probiotic Ricotta cheese matrix was observed for 12 days at refrigerated temperature. The reason for 12 days behaviour study of spoilage organisms in probiotic Ricotta cheese is due to the shelf life of probiotic Ricotta was estimated as 12 days of refrigerated storage based on sensory, physico-chemical, and textural quality as against 8 days for control Ricotta (Bhagwat *et al*., 2020b). The initial probiotic count was 7.87 log CFU/g. A significant ($p<0.05$) reduction was observed in La 05 (NCDC-291) count, at the end of 12 days of refrigerated storage. The final count of *L. acidophilus* La 05 (NCDC 291) at the end of 12 days of refrigerated storage was 7.6 log CFU/g (Bhagwat *et al*., 2020a).

The behaviour of *E. coli* in the control and probiotic Ricotta cheese matrix during 12 days of storage at 7°C is summarized in Fig. 3A. After inoculation, the initial count of the *E. coli* in control and probiotic cheese was 5.43 log CFU/g and 5.40 log CFU/g, respectively. In control cheese, the count of *E. coli* decreased non-significantly ($p>0.05$) to 5.23 log CFU/g and 4.96 log CFU/g, respectively on the 6th and 12th day of storage. In the probiotic Ricotta cheese matrix, the counts of *E. coli* were reduced to 4.90 log CFU/g and 4.50 log CFU/g, respectively on the 6th and 12th days of storage. At the end of the 12th day of the storage in the probiotic Ricotta cheese matrix, a significant ($p<0.05$) reduction in the population of the *E. coli* accounting for a 20% drop in the initial count was observed. However, the complete reduction of the test organism was not observed and the count was above 4 log CFU/g. Similar results were reported by various workers in probiotic Domiati cheese (El Kholy *et al*., 2014) and probiotic semi-hard goat cheese (Rolim *et al*., 2015). Most *E. coli* had been reported to withstand low pH and high temperature commonly associated with cheese manufacturing (Delbes-Paus *et al*., 2013). This indicates that probiotics remain effective to provide an inhibitory effect against spoilage organisms during storage. The inhibition rate obtained was 6.67% and 9.47%, respectively on the 6th and 12th day of storage against *E. coli*. Pathogenic *E. coli* O157:H7 count was not detected in probiotic Domiati cheese after 28 days of storage (El-kholy *et al*., 2014). However, the study was discontinued in this case considering the short shelf life of probiotic Ricotta cheese. *E. coli* O157:H7 was not eliminated in goat cheese prepared by using raw milk (Vernozy-Rozand *et al*., 2005).



[Values with different superscripted letters in a graph differs significantly ($p<0.05$). NCDC 291: *Lactobacillus acidophilus* La 05; NCDC 610: *Lactobacillus rhamnosus*; NCDC 400: *Lactobacillus fermentum*; NCDC 605: *Lactobacillus fermentum*; NCDC 22: *Lactobacillus reuteri*]

Fig. 3. Zone of inhibition of various probiotic organisms against various spoilage organisms; A: *S. aureus*; B: *E. coli*

The initial count of *S. aureus* was 5.32 log CFU/g, which was increased non-significantly ($p>0.05$) to 5.46 log CFU/g followed by decreased significantly ($p<0.05$) 4.73 log CFU/g, respectively on the 6th and 12th day of storage in control Ricotta matrix. The *S. aureus* count lowered significantly ($p<0.05$) to 4.18 log CFU/g from an initial 5.46 log CFU/g after 12 days of storage in probiotic Ricotta cheese. In the presence of the probiotic organism, a significant ($p<0.05$) reduction (23.4%) in *S. aureus* count was observed. The respective rate of reduction observed was 5.14% and 11.62% on the 6th and 12th days of storage. The behaviour of *S. aureus* in control and probiotic cheese during refrigerated storage in the presence and absence of *L. acidophilus* La 05 is summarized in Fig. 3B. Similar results of *S. aureus* inactivation were reported by several workers during ripening of cheese containing *L. plantarum* (Stecchini *et al*., 1991); cheese manufactured by pediocin producing *L. lactis* (Rodriguez *et al*., 2005); white brine cheese prepared with *L. acidophilus* (Sadeghi *et al*., 2013) and semi-hard goat cheese with *L. rhamnosus* (Rolim *et al*., 2015).

DISCUSSION

In the presence of probiotic organisms, a significant ($p<0.05$) reduction in the *E. coli* (20%) and *S. aureus* (23.4%) count was detected at the end of 12 days of refrigerated storage. Growth retardation of these spoilage organisms might be attributed to the presence of probiotic cells. It is a known fact that probiotic organisms can inhibit the undesirable organism *via* several mechanisms like competition for nutrients, production of antimicrobial elements like organic acids (lactic acid, acetic acid, etc.), hydrogen peroxide and bacteriocin (Tejero-Sarinena *et al*., 2012). Recent inhibition mechanism of probiotic organism has been suggested to the production of quorum signaling molecule responsible for the production of above mentioned antimicrobial substances (Quintieri *et al*., 2021). They may behave as antimicrobial peptides directly destroying other bacteria, as a signalling molecule through quorum sensing and cross-talk with bacterial communities (Dobson *et al*., 2012). In a study on fermented milk, furanone namely 2(5H)-furanone and bromo furanone produced by probiotic bacteria reduced acyl homoserine lactone of *Pseudomonas* spp. and increased the shelf life of the product up to 9 days by lowering the spoilage organisms (Shobharani and Aggarwal, 2010).

Probiotic organism *L. acidophilus* La 05 was able to inhibit spoilage organism *E. coli* and *S. aureus* in the Ricotta matrix. The inhibition rate against *E. coli and S. aureus* obtained was 9.47% and 11.62%, respectively, at the end of 12 days of storage. Inactivation of these spoilage organisms might be attributed to the presence of probiotic cells.

Conflict of interest: Authors have no conflict of interest in this study.

Author's contribution: SB: Conducted experiments and contributed in writing of manuscript; SG: Contributed in conceptualization and designing of project, further she contributed in modification of manuscript; YK: Contributed in designing of experiments and data analysis; PNR: Contributed in reviewing and modification of manuscript, language editing, data analysis and management.

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