




Production of probiotic Mozzarella cheese by incorporating locally isolated *Lactobacillus acidophilus*

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Abstract

Purpose: The present study was conducted to isolate and screen the potential probiotic strains for incorporation in Mozzarella cheese.

Methods: Probiotic cultures were isolated from different randomly purchased yogurt samples and were identified as *Lactobacillus* sp., *Bifidobacteria* sp., and *Pediococcus* sp. after morphological and biochemical characterization. Heat tolerance of isolates was tested at 55 °C and 65 °C to determine the survival of isolates in conditions similar to commercial cheese production. *Lactobacillus acidophilus* (S2) showed remarkable heat tolerance among all strains and was therefore selected to assess the probiotic potential. It showed good survival at acidic pH values (2–3). Moreover, it also showed > 50% tolerance to bile salt and was resistant to antibiotics, chloramphenicol, tetracycline, gentamycin, and vancomycin and also exhibited anti-microbial activity against *Salmonella typhimurium*, *Escherichia coli*, and *Staphylococcus aureus*. Thus, heat-tolerant *Lactobacillus acidophilus* (S2) isolate was an ideal strain for incorporation in Mozzarella cheese as probiotics. Three types of cheeses viz., cheese A with free cells of *Lactobacillus acidophilus* (S2), cheese B with encapsulated cells of *Lactobacillus acidophilus* (S2), and control cheese having no probiotics, were made.

Result: Microbiological analysis of prepared cheese revealed lesser loss of *Lactobacillus acidophilus* (S2) from encapsulated form (3.41×10^8 CFU/mL) compared to free cells of *Lactobacillus acidophilus* (S2) (1.10×10^7 CFU/mL). Coliforms were observed in control cheese after 10 days of storage, whereas no coliforms were observed in cheese A and cheese B even after 15 days of storage. Organoleptic properties of cheese A and cheese B were almost the same with an acceptability score of 2.7 ± 0.1 and 2.65 ± 0.1 , respectively. Control cheese got the lowest scores after 15 days of storage.

Conclusion: The addition of probiotics in cheese not only prolongs the shelf-life of cheese but also increases the organoleptic properties of the cheese, making cheese a good delivery system for probiotics.

Keywords: Probiotics, *Lactobacillus*, Yogurt, Quality assessment, Cheese

Introduction

Probiotic food products are gaining popularity among consumers due to health benefits. Such foods are not only nutritionally dense but also decrease the risk of different diseases (Mahrous et al. 2014). According to

FAO (Food and Agriculture Organization of the United Nations) “Probiotics are living microorganisms which, when administered at adequate levels (10^6 to 10^7 CFU/g), confer health benefits to the host” (FAO/WHO 2006). Thus, the therapeutic benefits of these products increase the consumption of probiotics (Weichselbaum 2009). Probiotics have been associated with the control of gastrointestinal and urinary tract infections. Other benefits include improvement in lactose tolerance,

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reduction in serum cholesterol level, enhancement of host's immunity, and prevention of colon cancer antibiotic-associated diarrhea and different allergic diseases (Bai and Ouyang 2006; Falagas et al. 2006; Krämer and Bischoff 2006; Sanders et al. 2007; O'Flaherty and Klaenhammer 2010; Yerlikaya 2014).

A majority of probiotics are bacteria, but yeasts may also be considered as probiotics because of their potential to survive in the digestive system and different health benefits to the host (Anal and Singh 2007; Senter et al. 2015). These health benefits are due to competition with pathogenic microbes, improvement in the host's immune system, production of acids, and antimicrobial proteins (Chen and Chen 2007). Probiotics are orally administered and are either incorporated in food products or in different non-food preparations, such as tablets, capsules, or sachets. Probiotic foods include dairy products, such as cheese, ice cream, dairy desserts, and yogurt, as well as non-dairy products, such as juices, cereals, and chocolates. Food products containing probiotic bacteria are known as "functional foods." Fermented functional foods have numerous health benefits due to incorporated probiotic microorganisms (Gobbetti et al. 2010). Viability of probiotic bacteria in a food product is influenced by many factors, such as acidity, post acidification, oxygen level of the product, penetration of oxygen through packaging, shortage of nutrients, and sensitivity to different antimicrobial substances produced by starter culture (Fortin et al., 2011).

Safety aspects include the origin of probiotic bacteria and the non-pathogenic nature of the strains. Functional aspects are related to survivability under the acidic conditions of human gastrointestinal tract and the ability to tolerate bile salts. Probiotics must attach and colonize to gastrointestinal epithelia and colonize there. They may also possess anti-carcinogenic and anti-mutagenic properties. Immunostimulation and antagonistic activity are some other functional aspects of probiotics. Good sensory properties, ability to be manufactured while staying viable and functional in the product, and survivability during storage till the end of shelf-life of the product without creating bad taste or textures are different technological aspects (Ortakci 2012; Senter et al. 2015).

There is an increase in the popularity and acceptance of functional foods throughout the world as they exert a positive impact on human health (Mattila-Sandholm et al. 2002). There is 7 to 32% increase in sales of different probiotic products each year on the basis of geographical locations and functions of probiotics (Helene et al. 2011). Mozzarella cheese is a prominent member of pasta filata cheeses, originating from Italy. Pasta filata cheeses are famous for their exceptional stretchability, shredability, and meltability which are due to unique texturing and plasticizing treatments of curd in hot

water. It is made by chemical acidification or biological acidification using commercial starter cultures, such as *Streptococcus thermophilus* (Minervini et al. 2012).

Many challenges are involved in the production of probiotic Mozzarella cheese, such as survival of probiotic bacteria during cheese production and storage (Fortin et al., 2011) while maintenance of sensory characteristics of cheese is another challenge. Therefore, there is a need to incorporate thermophilic probiotic strains with good sensory attributes. Therefore, the present study was undertaken to isolate the potential probiotic strains and screen them for their optimal efficacy, followed by incorporation in the Mozzarella cheese. As per available literature, it is a first ever report on the production of probiotic Mozzarella cheese by incorporation of a probiotic strain of *Lactobacillus acidophilus*.

Materials and methods

Isolation of microorganisms

A total of ten separate samples of fresh traditional yogurts were collected from local markets of Lahore, Pakistan, and stored at 4 °C to avoid contamination and spoilage during transportation (Astashkina et al. 2014). Samples were immediately processed for microbiological analysis in the lab after collection. For isolation of microorganisms, we followed the protocol as described by Karami et al. (2017) with some modification. One gram of each sample was inoculated in 10 ml of de Man, Rogosa, and Sharpe (MRS) broth tubes aseptically and incubated at 37 °C for 48 h under anaerobic conditions. Anaerobic conditions were provided by anaerobic jar with a gas pack. Pour plate technique was used for getting well-isolated colonies from cultures. After incubation, serial dilutions of MRS broth were made in 0.1% peptone water. One hundred microliter of each dilution was poured on plates containing molten MRS agar medium [MRS broth (55 gL⁻¹), L cysteine (0.5 gL⁻¹), and agar (55 gL⁻¹)]. All plates were incubated at 37 °C for 48 h under anaerobic conditions. Colonies were purified by streaking repeatedly on MRS BPB agar [MRS broth (55 gL⁻¹) + L cysteine (0.5 gL⁻¹) + Bromophenol blue (0.02 gL⁻¹) + agar (55 gL⁻¹)] plates. Pure cultures were stored at 4 °C for further use.

Physiochemical characterization of isolates

Biochemical characterization of isolates was carried out through catalase test, indole test, gas production, sugar fermentation profile, CaCO₃ utilization, and heat tolerance check (Holt et al. 1994). For the indole test, overnight culture (1%) was added in 5 ml of tryptophane broth and incubated at 37 °C under anaerobic conditions. After 24 h, 5 drops of Kovac's reagent were carefully added in tryptophane broth tubes and observed for the formation of a reddish-purple ring (Benson

2002). Likewise, the fermentation behavior of isolates was evaluated by observing gas production. Cultures that did not produce gas within 48 h were considered to be homofermentative, while those which produced gas were considered heterofermentative (Barakat et al. 2011). Bromothymol blue broth (pH 7) was used to determine the fermentation profile of isolates with different added carbohydrates (glucose, fructose, sucrose, lactose, maltose, and mannitol) (Naeem et al. 2012), whereas CaCO_3 utilization of isolates was observed on MRS agar with CaCO_3 (0.8 g/100 ml). Heat tolerance studies of the isolates were carried out by treating the cells at 65 and 55 °C for 10 min and then culturing on MRS agar plates (Minervini et al. 2012).

Physiological characterization of heat-resistant isolates was executed by evaluating NaCl tolerance, variation in pH of inoculated skimmed milk, and phenol tolerance. NaCl tolerance of isolates was determined using various concentrations of NaCl (1–10%) in MRS broth tubes inoculated with microorganisms. After 24 h, the bacterial count was determined by using a spectrophotometer (Hoque et al. 2010). For evaluation of variation in pH of skimmed milk, 10% sterilized skimmed milk tubes were inoculated with 1% (v/v) overnight fresh culture, then incubated for 24 h, and readings were taken after 0, 3, 6, 9, and 24 h using pH meter. For the measurement of phenol tolerance, phenol (0.4%) was added in MRS broth and inoculated with 1% (v/v) overnight fresh culture and incubated to check the inhibitory effect of phenol on isolated bacteria (Hoque et al. 2010).

Assessment of probiotic potential of isolates

The assessment of the probiotic potential of the selected isolate was performed by measuring acid tolerance, bile salt tolerance, antibiotic resistance, antimicrobial activity, and hemolytic activity (Pancheniak and Soccol 2005). To measure acid tolerance, *Lactobacillus* isolate was cultivated in MRS broth for 24 h at 37 °C under anaerobic conditions. Cells were harvested and cultured in MRS broth tubes with different pH values, such as 1, 2, and 3 pH units. After 1, 2, and 4 h of incubation, bacterial suspension was diluted in 0.1% peptone water and was spread on MRS agar plates. Plates were incubated at 37 °C under anaerobic conditions for 48 h, and percent survival was determined (Patil and Vishwanath 2012).

For measuring bile salt tolerance, the potential probiotic isolate was cultivated in MRS broth supplemented with 2% bile salt at 37 °C under anaerobic conditions for 48 h. Survival of the isolate was determined in terms of colony-forming units per milliliter (Patil and Vishwanath 2012). To assess the antibiotic resistance of the isolate, 100 μL of overnight culture was spread on the MRS agar plate and incubated for 2 h at 37 °C. Then, antibiotic discs were placed on the plate and incubated under the

same conditions for 48 h. The zone of inhibition was determined, and isolate was referred as sensitive, intermediate, or resistant according to the size scale of antibiotics (Naeem et al. 2012). The anti-microbial activity of isolate was determined by using the well diffusion method (Shylaja et al. 2010). For hemolytic activity, blood agar plates [Nutrient broth (13 gL^{-1}) + agar (15 gL^{-1}) + sheep blood (5%)] were freshly prepared and streaked with the selected isolate and incubated at 37 °C for 48 h under the anaerobic conditions. For beta hemolysis, zone formation was observed around the colonies. *Staphylococcus aureus* was used as a positive control.

Microencapsulation of isolates

A culture of the selected isolate was microencapsulated in alginate system using a method described by Sheu and Marshall (1993) with some modification. Cells of selected isolate ($\sim 10^{10}$ CFU/mL) was harvested by centrifugation at 10,000 rpm for 10 min and washed twice with 0.1% saline water. The pellet was then added in the same amount of 4% (w/v) sodium alginate solution and mixed thoroughly for 30 min at 4 °C using a magnetic stirrer. Thereafter, using a 10 mL sterile syringe, alginate and bacterial mixture was added drop by drop in 0.1 M CaCl_2 solution. After 10 min, beads were collected on filter paper and washed with saline water, blot dried with sterile filter paper, and stored at 4 °C.

Cheese production

Pasteurized milk was supplemented with 1% of *Streptococcus thermophilus* along with rennet and mixed well. Then, the selected probiotic isolate was added in cheese in encapsulated and free form ($\sim 10^{10}$ CFU/g) and mixed well for 2 min. Three types of cheeses, cheese A, cheese B, and control cheese, were made. In control cheese, no probiotic was added, while in cheese A free cells of the probiotic organism were added, and in cheese B encapsulated cells of the probiotic strain were added. Then, cheeses were allowed to coagulate for 60 min until the curd got separated from whey. Curd was collected, broken, and laid over a table to drain for 20 min. Hot brine (70 °C) was added to curd and mixed well until a homogenized paste was obtained. At 6 °C, the curd was added in iced brine (12%) for 6 h. After 4 h, the curd was dried, and cheese was packed in sterile plastic bags and stored at 4 °C for further analysis (Suliman et al. 2012).

Microbiological analysis of cheese

One gram of cheese was added in 10 mL of 0.1% peptone water and mixed well at 250 rpm for 10 min. Serial dilutions were made, and the probiotic bacteria were isolated using the pour plate technique. Encapsulated bacteria were enumerated by using phosphate buffer,

diluted and plated on MRS BPB agar plates at 37 °C under anaerobic conditions for 48 h. All samples were also cultured on McConkey agar for 24 h under aerobic conditions (Ortakci et al. 2012).

Compositional analysis of cheese

Compositional analysis of the cheese was performed as per the standard method of the Association of Analytical Chemists (AOAC 2000). To measure fat content in butyrometer, 10 mL of sulfuric acid was added, followed by the addition of 3 mL distilled water at 60 °C. Cheese sample (10 g) was added in greaseproof paper and added in butyrometer; 5 ml of water and 1 ml of amyl alcohol were also added and mixed vigorously. The mixture was then centrifuged at 1300 rpm for 5 min. Hot water butyrometer was placed at 65 °C for 3 min with the stopper facing downward, and the reading was taken by measuring the fat column.

For pH determination, 10 g of cheese sample was mixed with 6 mL of water and mixed thoroughly and then subjected to pH measurement. Moisture content of cheese was determined by the oven dry method. For salt content, 5 g of cheese sample and 100 mL of boiling water were added in a 250-mL conical flask, swirled well, cooled till 55 °C, and then titrated against silver nitrate using potassium chromate as an indicator. Color change from pale yellow to buffered color was recorded as end-point. Salt content was measured according to this formula:

$$\text{Salt \% (w/w)} = 58.45 \times N \times V \times 100/W \times 100$$

where “N” is the normality of silver nitrate, “V” is the weight of silver nitrate, and “W” is the weight of the sample.

Organoleptic analysis

Sensory analysis was done using 4-point hedonic scale (0–3). Six-membered panel was selected to evaluate the sensory attributes of cheeses based on their interest. Cheeses were taken out of the refrigerator and kept at room temperature for an hour. Then, cheese was sliced and served randomly for organoleptic analysis. Sensory attributes of experimental cheese included flavor, creamy nature, texture, and sour taste. A score of 3 was assigned to each attribute as follows: preferable (3), acceptable (2), needs modification (1), and not acceptable (0) (Minervini et al. 2012).

Statistical analysis

The experimental data was analyzed statistically using one way ANOVA by the method of Snedecor and Cochran (1980) using a computer software CoStat 3.03 CoHort Software, Berkeley, CA 94701. Significance has been presented in the form of probability ($p \leq 0.05$) values.

Results and discussion

Isolation and identification of probiotic bacteria

Probiotic bacteria were isolated from different locally produced yogurt samples. Out of 22 picked colonies, ten isolates with distinct colors and shapes were selected for further morphological and biochemical characterization.

Properly isolated colonies were picked up and transferred to solid media to observe their colony and cultural characteristics as described in Table 1. Seven isolates exhibited colony characteristics similar to the genus *Lactobacillus* as they appeared round, star-like, spindle-shaped, triangular, filiform, and irregular. Two isolates showed resemblance to the genus *Bifidobacterium* as they were round, creamy, convex spindle-shaped, and very soft in consistency. One isolate was found to be *Pediococcus* as colonies appeared round, raised, and smooth with small dotted-structure. Colony characteristics were better observed on MRS BPB agar as compared to MRS agar. Colonies showed different colors and various sizes on MRS BPB agar, whereas on MRS agar colonies were difficult to differentiate due to same color and size. Colonies of *Lactobacilli* appeared light blue while colonies of *Bifidobacteria* appeared dark blue on MRS BPB agar. The development of specific media has been tried by many researches for selective isolation of lactic acid bacteria (LAB), such as MRS agar (De Man et al. 1960), MRS clindamycin agar (Lankaputhra and Shah 1996), and LPSM (Bujalance et al. 2006). These media were appropriate to isolate specific LAB but not for isolation and selection of many lactic acid bacteria present as mixed culture in different foods. On MRS BPB agar, all LAB could easily grow, less incubation was required, and differentiation was easy (Lee and Lee 2008).

All isolates were characterized morphologically by Gram's staining, endospore staining, and motility test. They were found to be gram-positive and non-spore forming, and their morphological characteristics have been described (Table 2). Seven isolates resembled

Table 1 Colony characteristics of isolates

No	Isolates	Colony characteristics
1	S1	Round, convex, and smooth colonies
2	S2	Round, raised with entire margin colonies
3	S3	Round, flat smooth, and filiform colonies
4	S4	Round and flat colonies
5	S5	Round, regular, and smooth colonies
6	S6	Round and convex colonies
7	S7	Raised, small dot type colonies
8	S8	Round and raised colonies
9	S9	Triangular and raised colonies
10	S10	Round and raised spindle shaped colonies

Table 2 Morphological characteristics of isolates

Sr. no	Isolates	Gram's staining	Endospore staining	Motility test	Cell shape
1	S1	+	–	–	Thin rod shaped cells, branched, and v and Y arrangement in chains
2	S2	+	–	–	Thin rod shaped cells in chains with square ends
3	S3	+	–	–	Thin rod shape cells in long chains
4	S4	+	–	–	Regular rod shape cells in short chains
5	S5	+	–	–	Single rods
6	S6	+	–	–	Club shaped
7	S7	+	–	–	Cells spherical, tetrad, also in pairs
8	S8	+	–	–	Rod shape cells
9	S9	+	–	–	Rod shape cells as pairs
10	S10	+	–	–	Rods

+ Positive, – Negative

morphological characteristics of *Lactobacilli*, namely, S2, S3, S4, S5, S8, S9, and S10. Two isolates, namely, S1 and S6, were found to typically resemble the genus *Bifidobacterium*, i.e., gram-positive rods in “V” and “Y” arrangements, and club-shaped cells were observed under the microscope. Among them, one isolate, S7, resembled *Pediococcus* as cells were gram-positive diplococci and tetrad in arrangement. All the isolates were non-motile as they showed growth in stab line in spite of making whole media turbid.

LAB, especially *Lactobacilli*, are found in the gastrointestinal tract of human beings and animals. They are also present in milk and other dairy products (Jose et al. 2015). Hoque et al. (2010) isolated gram-positive non-spore-forming and non-motile *Lactobacillus* from yogurt, such as S2, S3, S4, S5, S8, S9, and S10 isolates. Zinedine and Faid (2007) isolated and characterized different *Bifidobacteria* from different sources, such as fermented foods, bovine meat, and fecal matter. They found that *Bifidobacteria* were in “V” and “Y” shape

arrangements and were gram-positive. In the present study, S1 and S6 isolates showed similar characteristics. Sukumar and Ghosh (2010) isolated *Pediococcus* which had gram-positive cocci-shaped cells arranged in tetrads from khadi, a traditional fermented food; S7 showed similar characteristics and belonged to the genus *Pediococcus*.

All the purified strains were subjected to different biochemical tests. They were catalase-negative, showing that the catalase enzyme was absent in all isolates. None of the isolates produced indole, indicating that they were all indole-negative as well (Table 3). All isolates were homofermentative in nature as no gas production was observed in Durham's tubes. The ability of isolates to ferment different sugars was also observed by change of media color from blue to yellow. Isolates S1 and S2 were able to ferment all sugars, such as glucose, sucrose, fructose, maltose, and lactose except for fructose; isolate “S7” showed the same fermentation as “S2” except for mannitol

Table 3 Biochemical characterization of isolates

No	Isolates	Catalase	Indole	Gas production	Glu	Fruc	Suc	Mani	Malt	Lact
1	S1	–	–	–	+	–	+	+	+	+
2	S2	–	–	–	+	+	+	+	+	+
3	S3	–	–	–	+	+	+	+	+	+
4	S4	–	–	–	+	+	–	+	+	+
5	S5	–	–	–	+	+	–	–	+	+
6	S6	–	–	–	+	+	+	–	+	+
7	S7	–	–	–	+	+	+	–	+	+
8	S8	–	–	–	+	+	–	+	+	+
9	S9	–	–	–	+	+	+	–	+	–
10	S10	–	–	–	+	+	+	+	+	+

Glu glucose, Fruc fructose, Suc sucrose, Mani mannitol, Malt maltose, Lact lactose

+ Positive, – Negative

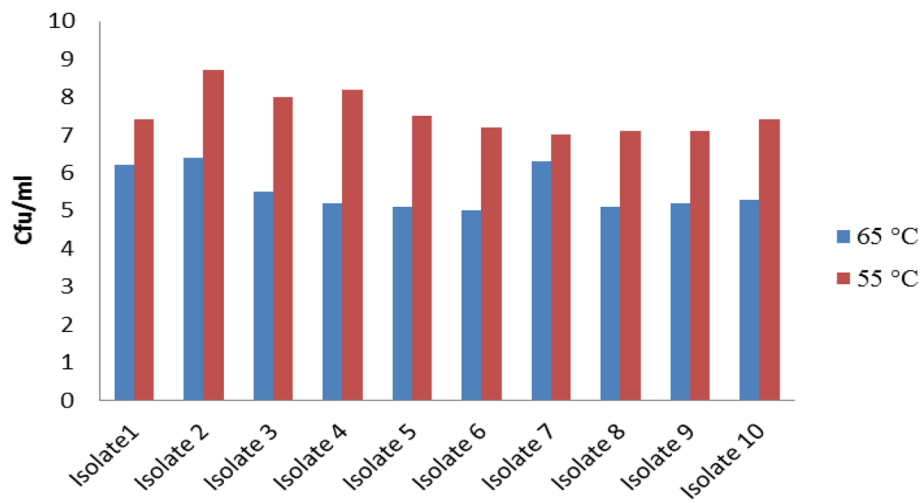


Fig. 1 Screening of the bacterial isolates for heat resistance

fermentation. All of the LAB were able to hydrolyze CaCO_3 by forming clear zones around their colonies. *Lactobacilli* and *Bifidobacteria* are catalase-negative, do not produce indole and gas from glucose as described by Hoque et al. (2010). Sukumar and Ghosh (2010) found that *Pediococcus* sp. showed the same biochemical behavior as shown by isolate “S7,” so it belonged to *Pediococcus* sp. Isolates belonging to the genera *Lactobacillus*, *Bifidobacterium*, and *Pediococcus* were also confirmed by fermentation behavior of isolates as described by Karna et al. (2007). Hence, isolates presumably belonged to the genera *Lactobacillus*, *Bifidobacterium*, and *Pediococcus* as indicated by morphological and biochemical characterization.

The most potent isolate (S2) based on different probiotics attributes was further subjected to identification

based on 16S rRNA gene sequencing and was identified as *Lactobacillus acidophilus* (data not shown here).

Screening for heat resistance

Heat tolerance of the isolates at 55 °C and 65 °C was observed, and it was found that they showed marked variation (Fig. 1). Survival rates of isolates were higher at 55 °C as compared to 65 °C. Among all the isolates, *Lactobacillus acidophilus* (S2) isolate showed good heat tolerance making it the most appropriate strain for incorporation in Mozzarella cheese. Ding and Shah (2007) also reported that heat treatment at 65 °C was lethal to all tested lactic acid bacteria. In another study, Minervini et al. (2012) screened 18 probiotic strains for their heat tolerance and got two thermophilic strains that were able to survive heat treatment at 65 and 55 °C for 10

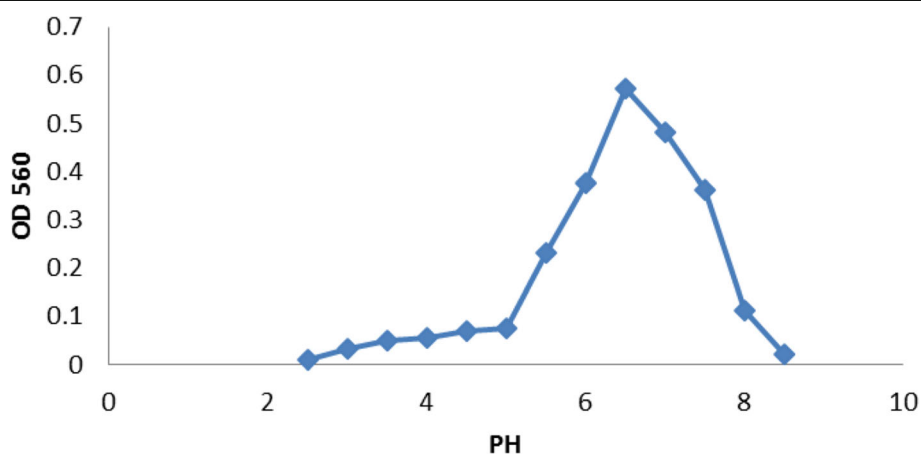


Fig. 2 Optimum growth pH of isolated *Lactobacillus acidophilus* (S2)

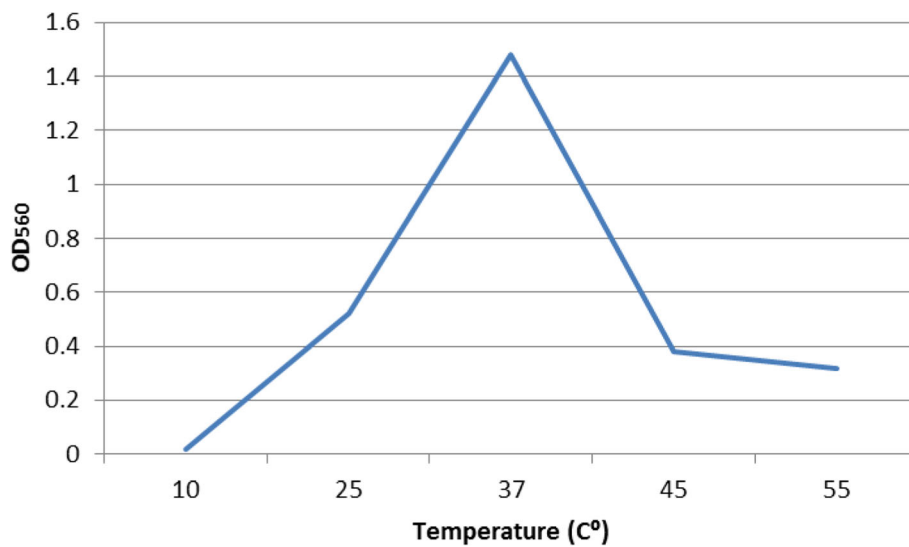


Fig. 3 Optimum growth temperature of isolated *Lactobacillus acidophilus* (S2)

min. Screening of heat-resistant probiotic strain is important for its use in hot stretched cheeses, as stretching of curd is done in hot brine.

Physiological characterization of *Lactobacillus acidophilus* (S2)

Heat-resistant *Lactobacillus acidophilus* (S2) was further characterized by physiological parameters. For this purpose, growth at different pH and temperature conditions was studied. Growth in the presence of 1–10% NaCl, 0.4% phenol tolerance, and pH variation in skimmed milk during 24 h of incubation was also analyzed.

The growth of bacteria changes dramatically as pH changes. Therefore, the growth of *L. acidophilus* was observed at different pH values between 2 and 8.5 to determine if it could grow under acidic as well as alkaline pH

levels. Optimum growth of the strain was observed at pH 6.5 (Fig. 2). Previously, Hoque et al. (2010) had reported that *Lactobacilli* can grow in acidic as well as alkaline pH and observed maximum growth of *Lactobacillus* isolates between pH values of 5 and 6.

For determining optimal growth temperature, growth at different temperatures, such as 10, 25, 37, 45, and 55 °C was observed. It was found that optimum temperature for growth of *Lactobacillus acidophilus* (S2) was 37 °C as maximum growth was observed at that temperature; *Lactobacillus acidophilus* (S2) was able to survive at 45–55 °C, exhibiting thermophilic behavior while no growth was observed at 10 °C (Fig. 3). Patil and Vishwanath (2012) reported that a *Lactobacillus* sp. isolated from cheese showed maximum growth at 37 °C although growth was also observed at 45 °C.

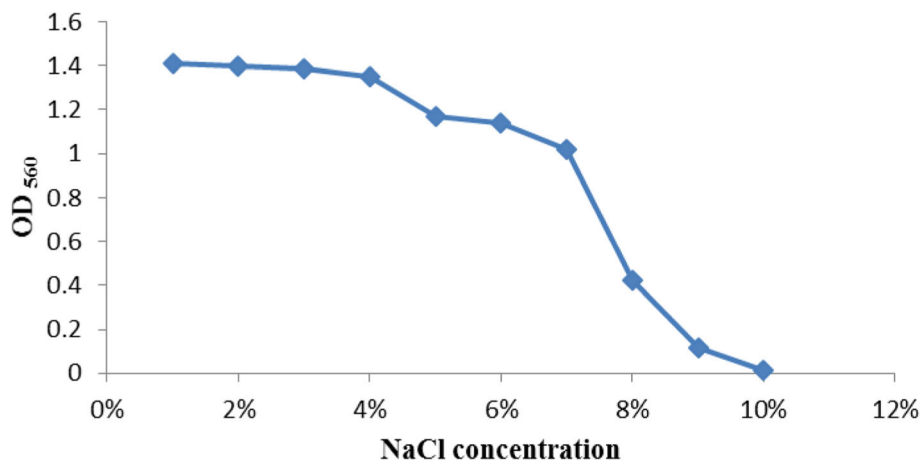


Fig. 4 NaCl tolerance profile of the isolated *Lactobacillus acidophilus* (S2)

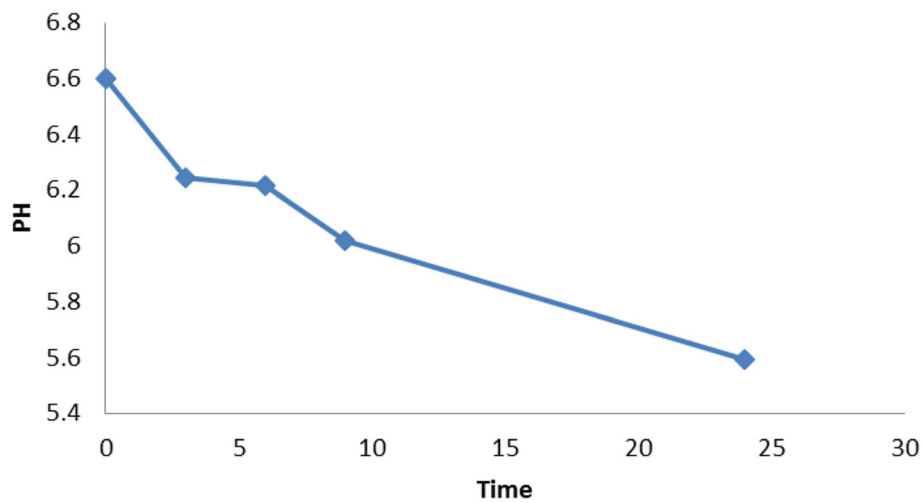


Fig. 5 Variation in skim milk's pH during incubation at 37°C

Tolerance to different NaCl concentrations by the isolate was also assessed as NaCl is an inhibitory substance in terms of the growth of microbes. It was found that *Lactobacillus acidophilus* (S2) was able to tolerate high concentrations of NaCl ranging from 1 to 9%. At 1% concentration of NaCl, very good growth was observed which gradually declined to no growth at 10% NaCl concentration (Fig. 4). Pancheniak and Soccol (2005) isolated a potential probiotic *Lactobacillus* sp. from the gastrointestinal tract of a swine which was able to tolerate NaCl concentration between 4 and 8%. Phenol has bacteriostatic activity and inhibits the growth of bacteria. For this purpose, the inhibitory effects of phenol on *Lactobacillus* sp. revealed that *Lactobacillus acidophilus* (S2) was a potential probiotic strain and could grow in

the presence of 0.4% phenol (data not included). Similar findings were previously reported by Hoque et al. (2010).

Lactobacillus acidophilus (S2) was inoculated in skimmed milk to elucidate the pH variation of skimmed milk with time. It was discovered that the pH of the skimmed milk decreased with an increase in time of incubation, and this was due to organic acid production. The pH of skimmed milk decreased from 6.6 to 5.5 pH units after 24 h of incubation (Fig. 5). Hoque et al. (2010) studied the pH variations in skimmed milk inoculated by *Lactobacilli* and reported that after 24 h, pH of skimmed milk changed from the initial 6.6 to 5.09 pH units after 24 h of incubation. In the present study, *Lactobacillus acidophilus* (S2) also decreased the pH of skimmed milk.

Table 4 Acidic pH tolerance of *Lactobacillus acidophilus* (S2)

pH	Time (hours)	Control	<i>L. acidophilus</i> (isolate S2)	Survival percentage (%)
1	1	231 (10^{-4})	–	0 ± 0
		231 (10^{-4})	–	
2	1	201 (10^{-4})	118	57.2 ± 3.5
		201 (10^{-4})	113	
	2	185 (10^{-5})	89	46 ± 3.5
		185 (10^{-5})	84	
	4	171 (10^{-6})	70	38.5 ± 5.6
		171 (10^{-6})	62	
3	1	256 (10^{-4})	186	71.8 ± 2.8
		256 (10^{-4})	182	
	2	153 (10^{-5})	101	64.05 ± 3.5
		153 (10^{-5})	96	
	4	198 (10^{-6})	69	36.36 ± 4.2
		198 (10^{-6})	75	

Each value is mean of three replicates; ± indicates the standard deviation from mean value; values are significant ($p \leq 0.05$)

Table 5 Antibiotic resistance of *Lactobacillus acidophilus* (S2)

Antibiotic	Concentration of antibiotic (μg)	Response
Chloramphenicol	30	R
Erythromycin	5	R
Tetracycline	5	R
Oxacillin	1	R
Vancomycin	30	R

R resistant

Assessment of probiotic potential

Lactobacilli strains are the most proclaimed potential probiotics (FAO/WHO 2002) and are widely used in food and non-food preparations.

Tolerance to acidic pH is the first criterion to select probiotics. The effect of acidic pH on the *Lactobacillus acidophilus* (S2) revealed that it was able to tolerate pH levels of 2 to 3, while no growth was observed at pH 1.0 (Table 4). Tolerance to acidic pH and bile salts is important for probiotic bacteria as they have to pass through the gastrointestinal tract and have exposure to bile salts in the intestine. Bile salt tolerance is the second most important criterion to select probiotics. *Lactobacillus acidophilus* (S2) showed good tolerance to 2% bile salt. Bile salt tolerance was 282 CFU/mL for control and 156 CFU/mL for *Lactobacillus acidophilus* (S2); the survival percentage was 55.3%. Corzo and Gilliland (1999) reported that the determination of survival of bacteria in acidic pH is a more accurate way to analyze their survival through the gastrointestinal tract. Bile salt tolerance is also important for probiotic bacteria as it helps probiotics to colonize the gastrointestinal tract (Pisano et al. 2014). Up to 0.3% is the average bile salt concentration faced by probiotic bacteria in the intestine which may reach to extremes. Pinto et al. (2006) reported that *Lactobacillus johnsonii* LA-1 showed better survival ability at acidic pH as compared to commercial *Bifidobacteria*.

Many lactic acid bacteria are resistant to different antibiotics. *Lactobacillus* sp. are referred to as sensitive to antibiotics when the zone of inhibition is ≥ 21 mm while ≤ 15 mm diameter zone of inhibition has been considered as resistant (Kacem et al. 2006). *Lactobacillus acidophilus* (S2) showed antibiotic resistance against chloramphenicol, tetracycline, gentamycin, and vancomycin (Table 5). Patil and Vishwanath (2012) reported

Table 6 Antimicrobial activity of *Lactobacillus acidophilus* (S2)

Test pathogens	Diameter of zone of inhibition (mm) \pm SD
<i>Salmonella thyphimurium</i>	11.50 \pm 1.0
<i>Escherichia coli</i>	9.00 \pm 0.3
<i>Staphylococcus aureus</i>	12.00 \pm 1.0

Each value is mean of three replicates; \pm indicates the standard deviation from mean value; values are significant ($p \leq 0.05$)

Table 7 Survival of *Lactobacillus acidophilus* (S2) in free and encapsulated form

Storage period (days)	Free <i>Lactobacillus</i> sp. (CFU/mL)	Encapsulated <i>Lactobacillus</i> sp. (CFU/mL)
1	1.10×10^7	3.41×10^8
5	2.64×10^7	2.34×10^8
10	2.10×10^7	2.12×10^8
15	3.21×10^7	2.54×10^8

that the antibiotic resistance of lactic acid bacteria can be therapeutic and preventive against different intestinal pathogens and would help to balance the normal intestinal flora of patients whose normal microbial flora have been disturbed due to intake of antibiotics. Among all antibiotics, vancomycin is the most important as it is one of the latest antibiotics that is broadly effective against clinical infections (Devriese and Butaye 1998). According to safety aspects, Mathur and Singh (2005) reported that an increase in antibiotic resistance in intestinal flora is alarming and might be due to the extensive use of antibiotics in animal foods.

The antimicrobial effect of *Lactobacillus acidophilus* (S2) was observed against *Salmonella thyphimurium*, *Escherichia coli*, and *Staphylococcus aureus* (Table 6). The diameter of the zone of inhibition against *Salmonella thyphimurium* was 10.00 ± 1.0 , while the zone of inhibition against *Escherichia coli* was 8.50 ± 0.3 . The selected isolate exhibited antimicrobial activity against enteric pathogens; therefore, it could be used for prevention of certain intestinal infections such as diarrhea. The findings of current study were in agreement with the previously documented studies (Pan et al. 2009).

Lactobacillus acidophilus (S2) was found to be non-hemolytic as there was no change in the color of blood agar around the *Lactobacillus acidophilus* (S2) colony. Thus, *Lactobacillus acidophilus* (S2) was non-hemolytic and was a potential probiotic bacterium. Lactic acid bacteria show antimicrobial activity against gastrointestinal pathogens due to the production of bacteriocins which are peptides that have antimicrobial activity (Liasi et al. 2009). Antimicrobial activity of lactic acid bacteria may be due to H_2O_2 , organic acids, bacteriocins, or inhibitory substances of metabolites (Testa et al. 2003). Zhang et al. (2008) reported that *Lactobacilli* exhibit good anti-microbial activity against intestinal pathogens. Ehrmann et al. (2002) reported that the anti-microbial activity is due to acid production which is a major factor. Fayol-Messaoudi et al. (2005) also revealed that antimicrobial activity of lactic acid bacteria is due to low pH caused by acid production. Patil and Vishwanath (2012) reported that in their study that *Lactobacillus* sp. was non-

Table 8 Safety assessment of probiotic cheese

Coliform count (CFU/mL)			
Storage period (days)	Control cheese (CFU/mL)	Cheese A (free <i>Lactobacillus</i> sp.) (CFU/mL)	Cheese B (encapsulated <i>Lactobacillus</i> sp.) (CFU/mL)
1	ND	ND	ND
5	ND	ND	ND
10	2.4	ND	ND
15	3.7	1.1	1.5

All three types of cheeses were stored at 4 °C
ND not detected

hemolytic; therefore, *Lactobacillus acidophilus* was found to be potentially safe to use as a probiotic.

Cheese production and characterization

Three types of cheese, cheese A, cheese B, and control cheese, were made: cheese A with free cells of *Lactobacillus acidophilus* (S2), cheese B with encapsulated cells of *Lactobacillus acidophilus* (S2), and control cheese with no added probiotic. These cheeses were stored at 4 °C and examined for microbiological, compositional, and organoleptic analysis.

Mozzarella cheese is a hot stretched cheese involving kneading treatment. Probiotic loss can occur during the production of cheese and for this reason $\sim 10^{10}$ CFU/mL of *Lactobacillus acidophilus* (S2) was added in milk after coagulation for maintaining good bacterial count in cheese. Encapsulated *Lactobacillus acidophilus* (S2) showed better survival as compared to free cells of *Lactobacillus acidophilus* (S2) (Table 7); the free count of *Lactobacillus acidophilus* (S2) was 1.10×10^7 CFU/mL, while encapsulated *Lactobacillus acidophilus* (S2) count was 3.41×10^8 CFU/mL in cheese. Less loss of encapsulated bacteria was noted as compared to free cells of *Lactobacillus acidophilus* (S2). During 15 days of storage at 4 °C, no loss in encapsulated and free *Lactobacillus acidophilus* (S2) was observed. No coliform was observed in cheese A containing free cells of probiotic strain and cheese B containing encapsulated *Lactobacillus acidophilus* (S2) as compared to control cheese (Table 8). Coliforms are common contaminants responsible for spoilage of Mozzarella cheese due to its high moisture and low salt content (Altieri et al. 2005; De Angelis et al. 2008; Sinigaglia et al. 2008). In cheese A and cheese B, the added probiotic bacteria did not allow the growth of coliforms due to their antimicrobial

activity as compared to control cheese. This may also have effect on the storage life of Mozzarella cheese. As previously reported, studies have also documented that the addition of probiotics in any food product, such as cheese and ice cream, increases the shelf-life of the product by suppressing the growth of coliforms mainly through bacteriocins production (Altieri et al. 2005; Minervini et al. 2012). Ding and Shah (2007) studied the effect of heat treatments on different lactic acid bacteria and reported that lactic acid bacteria tolerate harsher conditions in a better way when they are in encapsulated form. Ortakci et al. (2012) reported that bacterial count in hot stretched cheeses is high when bacteria are incorporated in encapsulated form as compared to free cells of bacteria because high temperature proves lethal for bacteria. According to safety aspects, it was found that the addition of probiotics increased the shelf-life of food products as *Lactobacillus* sp. did not allow the growth of coliforms due to their antimicrobial activity as has already been shown by Minervini et al. (2012).

Moisture content, pH, NaCl, and fat content of cheeses at day 1 during storage at 4 °C were determined. Moisture content of cheese A and cheese B was almost the same ($57.1\% \pm 0.05$) while the moisture content of control cheese was slightly higher, i.e., $60.1\% \pm 0.05$. The pH values of cheese A and cheese B were almost the same (5.1 ± 0.05 and 5.2 ± 0.05 , respectively) while the pH value of control cheese was a little high (5.5 ± 0.05). Fat content was low in cheese B (15.6 ± 0.1) as compared to cheese A (16.2 ± 0.1), while the fat content of control cheese was found to be 12.2 ± 0.1 . NaCl concentration was high in cheese B (1.5 ± 0.3) as compared to cheese A (1.3 ± 0.3) (Table 9). An increase in NaCl concentration in cheese containing microencapsulated probiotics might be because of alginate. As alginate was

Table 9 Moisture content, pH, NaCl, and fat content of probiotic cheeses at day 1

Cheese type	Moisture content (%)	pH	NaCl (%)	Fat content (%)
Control	60.1 ± 0.05	5.5 ± 0.05	1.1 ± 0.1	12.2 ± 0.1
Cheese A	57.1 ± 0.05	5.1 ± 0.05	1.3 ± 0.3	16.2 ± 0.1
Cheese B	57.1 ± 0.05	5.2 ± 0.05	1.5 ± 0.3	15.6 ± 0.1

Cheese stored at 4 °C; Each value is mean of three replicates; \pm indicates the standard deviation from mean value; values are significant ($p \leq 0.05$)

Table 10 Organoleptic analysis of probiotic cheese

Cheese types	Flavor	Creamy	Texture	Sour	Acceptability
Organoleptic analysis at day 1					
Control cheese	1 ± 0.6	1.1 ± 0.4	1.3 ± 0.5	0.8 ± 0.4	1.05 ± 0.2
Cheese A	2.2 ± 0.7	2.3 ± 0.5	1.7 ± 0.5	1.8 ± 0.4	2 ± 0.2
Cheese B	1.8 ± 0.4	2.2 ± 0.8	1.7 ± 0.5	2.1 ± 0.5	1.95 ± 0.2
Organoleptic analysis at day 7					
Control cheese	1.3 ± 0.5	1.5 ± 0.5	1.3 ± 0.5	1.5 ± 0.5	1.4 ± 0.1
Cheese A	2.8 ± 0.4	2.8 ± 0.4	2.6 ± 0.5	2.8 ± 0.4	2.7 ± 0.1
Cheese B	2.7 ± 0.5	2.5 ± 0.5	2.8 ± 0.5	2.4 ± 0.1	2.65 ± 0.1

Each value is mean of three replicates; ± indicates the standard deviation from mean value; values are significant ($p \leq 0.05$)

used in the present study for microencapsulation purpose and slightly high level of NaCl was found in cheese B containing probiotic in encapsulated form. This observation is comparable to Ortakci et al. (2012). Minervini et al. (2012) who had studied the gross composition of control and probiotic Mozzarella cheese had reported that the moisture content and pH of probiotic Mozzarella cheese were low as compared to control cheese. Similar findings have been obtained in the present study.

Organoleptic analysis of cheeses was done at 1st and 7th days of storage at 4 °C (Table 10). Cheese A and cheese B got the highest scores as compared to control cheese. The addition of probiotic bacteria enhanced the organoleptic properties of cheese. Overall, acceptability was same for cheese A and cheese B. Incorporations of probiotic in different types of cheeses have been studied by different researchers (Minervini et al. 2012; Shahab Lavasani et al. 2011; Albenzio et al. 2013). They found that the probiotic added cheeses possessed good sensory attributes such as flavor, aroma, and texture as compared to control cheese. Similar results were obtained in the present study. Gobetti et al. (2010) reported that the enhancement of flavor was mainly due to the formation of free amino acid and volatile compounds. Additions of probiotics in food products, such as cheese, not only prolong their shelf-life but also increase the organoleptic properties of cheese making it a good delivery system for probiotics.

Conclusion

This study revealed that *Lactobacillus acidophilus* was the most efficient strain for the production of probiotic Mozzarella cheese. The present study also documents that the probiotic supplemented cheese possessed good sensory attributes as compared to non-probiotic added cheese. The use of probiotics in cheese not only prolonged the shelf-life of the cheese but also increased the organoleptic properties of the cheese and thus making it a good delivery system for probiotics.

Research involving human participants and/or animals

Involvement of human participants.

Authors' contributions

HM conceived the idea and supervised the study. SY conducted the experiments and analyzed the data. SY and HM wrote the manuscript. IH reviewed and approved the final manuscript.

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Ethics approval and consent to participate

Ethical approval was taken from the Institutional Bioethics Committee, GCU Lahore, and written informed consent was taken from study participants.

Competing interests

All authors declare no conflict of interests.

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