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Evaluation of isolated *Lactobacillus* strains as Probiotics in yogurt preparation

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Abstract

Background: Probiotics are live organisms that when ingested in adequate amount are believed to provide health benefits to the host. Probiotics are when combined with prebiotics are termed as synbiotics and in that case prebiotics enables the probiotic organisms to survive better in the host. The main objective of this study is to investigate *Lactobacillus* strains isolated from dairy products with significant probiotic potential and their utilization in yogurt preparation.

Methods: Eleven (11) bacterial strains were identified as *Lactobacillus* by following Bergey's manual scheme. Antibacterial activity was checked by agar well diffusion method. Their ability to grow at different bile salt concentration, pH concentration and in the presence of pepsin enzyme was checked under *in vitro* condition. Finally, they potential strains were evaluated as probiotic starter in yogurt preparation.

Results: Out of total strains isolated, 52% strains were considered as *Lactobacillus*. Half of the strains (50%) showed antibacterial activity against selected pathogens and the best zone was formed by S4BM2 (15mm) against *Salmonella typhi*. All the isolated strains had the ability to grow in the presence of 0.2% bile salt concentration; at pH 3, pH5 and in the presence of pepsin enzyme. The efficacy of *Lactobacilli* strains as starter culture in yogurt was checked and found that yogurt processed with combination of S2Y2 with apple pieces showed significant results as compared to uninoculated yogurt.

Conclusion: It was concluded that strain i.e. S2Y2 can further be used in different dairy industries for yogurt processing to improve the quality of yogurt.



Introduction

The word "Probiotics" is derived from a Greek word in which "Pro" means favor and "Bios" means life. Probiotics are nonpathogenic microorganism and are beneficial for its host as they improve microbial load in gastrointestinal tract [1]. In 2001, expert consultation of international scientists were working on behalf of FAO and WHO debated on probiotics and defined it as live microorganisms that confer beneficial effects on host when administered in sufficient amount [2]. To be said to be a probiotic, a bacterium must meet several criteria including the ability to survive in the presence of bile salts and acids, produce antimicrobial compounds and colonize the intestines and resist antibiotics [3].

Non-digestible food ingredients that promote the activity and growth of probiotics are known as prebiotics as they benefit the health of host by carrying out beneficial activities in digestive tract and they help in the proliferation of healthy and beneficial microbes in the host thus making us healthy [4]. Currently prebiotics, probiotics and their combined action known as synbiotics holds a greater attention as they are involved in the enhancement of wellbeing of human by using natural sources [5]. Prebiotics concept has now expanded because of progress in research in the field of micro biome which increases our knowledge of composition of microbiota which enabled us to spot substance affecting colonization [6].

Lactic acid bacteria (LAB) are normal, physiological microflora of intestines, female genital tract and mouth and are also frequently present in vegetables, meat and dairy products, such as yogurt and milk [7]. A large number of lactic acid bacteria have been proposed and they have been used as probiotic strains. The lactic acid bacteria linked with food consists of 11 genera including *Pediococcus*, *Streptococcus*, *Leuconostoc*, *Lactosphaera*, *Carnobacterium*, *Lactobacillus*, *Vagococcus*, *Weissella* and *Oenococcus* [8]. Among them *Lactobacillus* species is most common and are compatible with human gastrointestinal system because of its instinctive resistance to bile, acid and interfering enzymes such pepsin and pepsinogen [9]. The bacterial species of lactic acid bacteria represents several benefits which are connected with the host [8].

Various health benefits provided by lactic acid bacteria have made them promising probiotic candidate [10]. LAB provides several health benefits like in susceptible individuals it reduces the risk of allergies and maximum bioavailability of nutrients. It also acts as a source of alleviation of some kind of intolerance. While there are several reports that show the hypocholesterolemic, antimutagenic, anti-osteoporotic, anticarcinogenic, immunomodulatory and antihypertensive impacts by these beneficial candidates [11].

Cultured dairy products such as yogurt are widely consumed as a healthy and nutritious food. Yogurt is complex gel system that incorporates lipids, polysaccharides and proteins in its structure [12]. Due to bioavailability of nutrients and high digestibility, yogurt is considered as a healthy food. This fermented milk is

recommended to a person with lactose intolerance, metabolic disorder and gastrointestinal disorders such as irritable bowel disease. This fermented milk i.e. yogurt also aids in immune function and weight control [13]. As lactic acid bacteria (LAB) played a vital role in nutrition and food processing, that's why most of the probiotics used by most of the food industries are LAB. Lactic acid bacterial strains are incorporated as starter cultures in fermented food products and they play beneficial and/or functional role in maintaining their metabolism such as capacities of metabolite production and substrate utilization. Natural cultures containing these bacterial strains were marketed results in increased acceptance of probiotics strains and products by consumers as they are helpful in digestion processes and health. Probiotic bacteria used in food products cover a large scale of population though they have a limited scope in therapeutic application [14].

Dairy industries are always in quest of novel probiotic and beneficial strains so that they can develop novel dairy products that can be offered to consumers as substitute to keep their health and well-being [15]. Since 1990's, fermented milk products containing probiotic strains in their composition have gained significant importance. Moreover, for facilitating the security verification of the products fermented, dairy products have a long safety usage [16]. For probiotic strains, fermented milk products are usually taken as ideal vehicle though other dairy products are also being used for this purpose [14].

The dominant benchmark in the selection of a potential probiotic strain are the ability to survive in GIT where bile and acids are present and also the ability to stick to the intestinal wall. Moreover, it is necessary that the candidate probiotic strains have the ability to manifest some enhanced digestive functions and antimicrobial activities against pathogens [17].

The significance of probiotics is well acknowledged, and everlasting efforts are being carried out to discover effective strains that are able to contribute specific functions in the host. Thus, the main aim of the study was to isolate the *Lactobacillus* strains with Probiotics properties for their significant use in the food industry. Moreover, this study was aimed to test the possibility of producing a probiotic yogurt by the addition of *Lactobacillus* enriched apple pieces and to check the effect of enriched fruits on the physiochemical properties of yogurt.

Methods

Isolation and identification of strains

Raw cow and buffalo milk and homemade yogurt were collected randomly from different dairy farms of Multan, Pakistan and were stored in sterile autoclaved containers at 4°C until delivery to the laboratory for isolation of *Lactobacillus* strains.

For isolation *Lactobacillus* strains samples were diluted and were allowed to spread them on solidified MRS (de Man, Rogosa and Sharpe) agar (Oxoid). Plates were then incubated at 37°C for 24-48 h under anaerobic condition. Colonies having different morphological appearance were selected [18]. Gram staining and

biochemical tests were done according to Bergey's Manual of Bacteriology scheme to identify *Lactobacillus* strains [19] and strains were selected according to their biochemical tests. Bacterial strains that showed Gram positive reaction, catalase negative reaction, were fermenters of different carbohydrates like glucose, sucrose; maltose, lactose and dextrose were considered as *Lactobacillus* and were selected for antimicrobial activity [20].

Evaluation of probiotic properties

Acid and bile salt tolerance of isolated strains

Isolated strains were investigated for bile salt tolerance and for that purpose; MRS broth was prepared with varying concentrations of oxgall bile salt (0.2% and 2%) and pH (pH 2, 5). After preparation of MRS broth, each isolate was transferred into tubes (triplicates) containing broth and tubes were incubated at 37°C for 24 h. After incubation, optical density (O.D) was measured against the control. Isolates showing resistance more than 50% at pH 2 were considered acid tolerant strains. The percentage resistance in both cases (bile/acid pH values) was calculated as follows [18].

$$\% \text{ Resistance} = \frac{\text{Increment of O.D in MRS broth with pH 2, 5 / Bile salt 0.2\%, 2\%} \times 100}{\text{Increment of O.D of control}}$$

Resistance to stimulated gastric juice

Stimulated gastric juice was prepared and was adjusted to pH 3 by using HCl. Resistance to stimulated gastric juice was checked according to Feng *et al.*, with slight modifications [21]. *Lactobacillus* strains were inoculated into MRS broth and were incubated at 37°C for 24 hours. Fresh cultures were harvested by centrifugation at 7000 x g for 10 min and suspended in saline solution. Dilutions were made to reach final dilution of 10⁹ CFU/ml. Thereafter, each cell suspension was mixed with stimulated gastric juice by vortex mixing and after that tubes were incubated at 37°C for 5 hours. Bacterial growth was estimated by taking optical density (O.D) at 560nm [21].

Physiological characterization

Physiological properties like ability of the strains to grow at different temperatures and different NaCl concentration were checked and the protocol is described briefly. MRS was inoculated with overnight cultures of selected bacterial strains and then these strains were grown at different temperatures i.e. 25°C, 37°C and 45°C and at three different concentrations of NaCl i.e. 1%, 3%, 6.5%. These different concentrations of NaCl were adjusted in MRS broth. After that the tubes were incubated for 24-48 hours [22].

Detection of antibacterial activity of selected strains

For antibacterial screening of isolated bacterial cultures, these cultures were inoculated in MRS broth and incubated at 37°C for 24-48 h on shaker to carry out fermentation process. 2 ml of the broth was taken in eppendorf and centrifuged at 14224 rcf for 10 min. After centrifugation, antibacterial activity of supernatant of strains was tested against test organisms by following the protocol of agar well diffusion method. Briefly, tested

microorganisms were spread evenly on solidified agar plates and were allowed to dry. Wells (each 7mm in diameter) on agar plates were made with the help of sterile borer following with loading of wells with 60 - 70 µl of isolated bacterial cultures filtrate (supernatant). Plates were then incubated at 37°C for 24 hours [22].

Yogurt processing

Processing of fresh apple

Fresh apples were purchased from the market and were cut into wedges with the help of stainless-steel knife while precautions were taken to avoid cross contamination [23]. Apples were selected because of their prebiotic efficacy enriched with probiotic strains.

Production of *Lactobacillus* enriched apples

Lactobacillus enriched apple was prepared by mixing fresh apple pieces with biomass of *Lactobacillus* and was incubated at 37°C for 48 hours without agitation. Then liquid was drained off and enriched apple was washed twice with Ringer's solution to remove free cells. The prepared enriched support was then freeze dried [23].

Yogurt production

For yogurt production, milk was dispensed equally in test tubes, pasteurized and was allowed to cool down at 45°C. The viability of yogurt starter culture was checked and was added in each part following the addition of *Lactobacillus* enriched apple pieces except the one which was used as a control [23]. For probiotic yogurt processing, pasteurized milk was cooled quickly to 45°C. After that yogurt starter cultures were added along with probiotic strains except in control part in which only yogurt cultures were added [24]. Tubes were then incubated at 37°C to 45°C until the pH was decreased to 4.6-4.8. The yogurt samples were then transferred to cold storage and stored at 4°C for 10 days. During that period different physicochemical properties were measured [24].

Determination of physicochemical properties

The pH value of yogurt and milk was measured by using the pH meter at 0 and 10 days of storage [12]. The total solid content of samples was determined by method described as follows; initial weight of sample was taken and then it is placed in hot dry oven at 110°C. After drying, samples were cooled at room temperature and final weight of sample was then measured. Final weight of sample after drying was considered as total solid contents. Total solid content was calculated by using following formula [23].

$$\text{Total solid content} = \text{initial weight of sample} - \text{final weight}$$

To determine syneresis, 3 tubes of 2ml of yogurt or fermented milk were weighed and placed in a centrifuge. Tubes were then centrifuged at 2,000 rcf for 5 min. After that separated serum was weighed. Syneresis was calculated using the following formula [24].

$$\text{Syneresis \%} = \frac{W_{sx} - 100}{W_y}$$

Where W_s was the supernatant weight after centrifugation and W_y was the weight of the yogurt or fermented milk in the tube.

Statistical analysis

Results of physicochemical properties i.e. pH, total solid content and syneresis were done in triplicates and are expressed as the mean and standard deviation of three determinations. Duncan test was used to check significant differences among different results were computed using IBM SPSS Statistics 22 software. A p value of <0.05 was considered as statistically significant for all analysis.

Results

Isolation and biochemical characterization of isolated strains

A total of 19 strains were isolated from different dairy products out of which 10 strains were considered as *Lactobacillus* strains on the basis of colony morphology and biochemical characterization. Morphological characterization revealed that all of the strains were pinpoint, round in shape and all strains have entire edges. According to biochemical characterization, all of the strains were negative for biochemical characterization including citrate test, mannitol salt agar test, VP test, methyl test and catalase test. The strains showed different sugar utilization and fermentation capabilities.

Probiotic characterization of isolated *Lactobacilli* strains

Acid and bile salt tolerance

All of the strains were able to grow at different concentration of bile salt i.e. 0.2%, 2% bile salt. Strain S4BM2 showed best resistance at 0.2% and 2% bile salt concentration (fig 1). All of the strains that were selected were able to grow at different acidic conditions i.e. pH2, pH5. Strains S4BM2 and S1BM4 showed best resistance at pH 2 and pH 5 respectively (fig 1).

Exposure to gastric stimulants

Different strains showed significant variability in growth in the presence of stimulated gastric juice with the best result being shown by S4BM2 that was 0.629 (fig 1).

Physiological characterization

5 strains that were selected were tested for their physiological characteristic i.e. ability of the strains to grow at different NaCl concentrations and at different temperatures.

All of the 5 selected strains showed greater resistance at different temperatures and showed best growth at 37°C. All of the strains were also able to grow at different NaCl concentration i.e. 1%, 3%, 6.5%.

All of the 5 selected strains were able to grow in the presence of different concentrations of NaCl and were also able to grow at different temperatures i.e. 37°C, 25°C and 45°C (fig 1).

Antibacterial activity of isolated strains

Antibacterial activity of selected *Lactobacillus* strains was checked against different pathogens including *Enterobacter* spp., *E. coli* (9473), *E. coli* (ETCC), MRSA

6, *Klebsiella* spp., *Salmonella typhi* and *P. aeruginosa*. Strains that showed best activities were S4BM2, S2Y2, S2Y1, S1BM4 and S4Y2 with the best zone (15mm) being shown by S4BM2 against *Salmonella* (table 1). On the basis of antibacterial activity of isolated strains against different selected pathogens, 5 strains were selected that gave best result against pathogenic strains.

Yogurt processing

Different physiological properties of yogurt samples were checked at 0 day and 10th day of storage. Yogurt formed with the combination of S2Y2 and apple pieces showed promising results in all the three categories i.e. syneresis, pH determination and total solid content. Yogurt processed with *Lactobacillus* strains combined with apple pieces showed significant results as compared to yogurt formed with *Lactobacillus* strains only with the best result being formed by S2Y2 combined with apple pieces (fig 2).

Discussion

Nineteen (19) strains were isolated from different dairy products out of which 10 strains were considered as *Lactobacillus* strains on the basis of colony morphology and biochemical characterization done according to Bergey's manual of Bacteriology scheme [19]. According to morphological characterization, all of the strains were round in shape and all strains have entire edges. Antimicrobial activity of 10 selected *Lactobacillus* strains was checked against different pathogens. For a strain to be of a good probiotic character, it is necessary that they should inhibit the growth of harmful microorganisms. Strains such as S4BM2, S1BM4, S4Y2, S2Y1, and S2Y2 showed viable zone of inhibition against different pathogens including *Enterobacter* spp., *E. coli* (9473), *E. coli* (ETCC), MRSA 6, *Klebsiella* spp., *Salmonella typhi* and *P. aeruginosa*. Maximum zone of inhibition was shown by S4BM2 (15mm) against *Salmonella* and same strain showed zone of inhibition (14mm) against *E. coli*. Study of Kumar and Kumar (2015) showed that only one strain (LBS2) inhibited the growth of *E. coli*, however, in our study it was shown that strains S4BM2, S1BM4, S4Y2, S2Y2, S2Y1 inhibited the growth of *E. coli* [18]. Five strains with best zones were selected for the evaluation of further probiotic properties. The growth of isolates at different temperatures (25°C, 37°C and 45°C) and different NaCl concentration (1%, 3% and 6.5%) confirms that isolated strains are *Lactobacillus*. Similar results were present in the study of Pundir *et al.*, [22]; they isolated strains from different food samples. In their study, all of the strains were also able to grow in the presence of different NaCl concentrations and at different temperatures. Main reason to select such temperature range (25°C, 37°C, and 45°C) was to find out whether the isolated strains were able to grow within normal body temperature range or not. NaCl was considered as an inhibitory substance so, it was necessary to check whether the strains were able to grow in the presence of different concentrations of NaCl concentrations or not [22]. Activity of probiotics was influenced by several gastrointestinal tract factors including low pH, pepsin and bile salt. For the strains to

Strains	Morphological characterization							Biochemical characterization				Sugar utilization				
	Size	Shape	Edge	Color	Opacity	Elevation	Surface	Catalase test	VP test	Citrate test	MSA test	Glu	Malt	Lac	Suc	Dex
S ₄ BM ₂	P	R	E	W	OP	R	S	-	-	-	-	-	+	+	+	-
S ₁ BM ₄	P	R	E	W	OP	C	S	-	-	-	-	+	+	+	-	+
S ₄ Y ₂	P	R	E	W	OP	R	S	-	-	-	-	-	+	+	+	+
S ₂ Y ₂	P	R	E	W	OP	R	S	-	-	-	-	+	+	-	+	+
S ₂ Y ₁	P	R	E	W	OP	C	S	-	-	-	-	-	+	+	+	+

Table 1: Morphological and biochemical characterization of selected strains by using Bergey's manual of Determinative Bacteriology Size: P (Punctiform, Shape: R (Round), Edges: E (entire), Color: W (white), OW (off white), Opacity: OP,(opaque),Elevation: R (raised), C (convex),Surface: S (smooth), VP: Voges Proskauer, MSA: Mannitol Salt Agar, Glu: Glucose, Malt: Maltose, Lac: Lactose, Suc: Sucrose, Dex: Dextrose

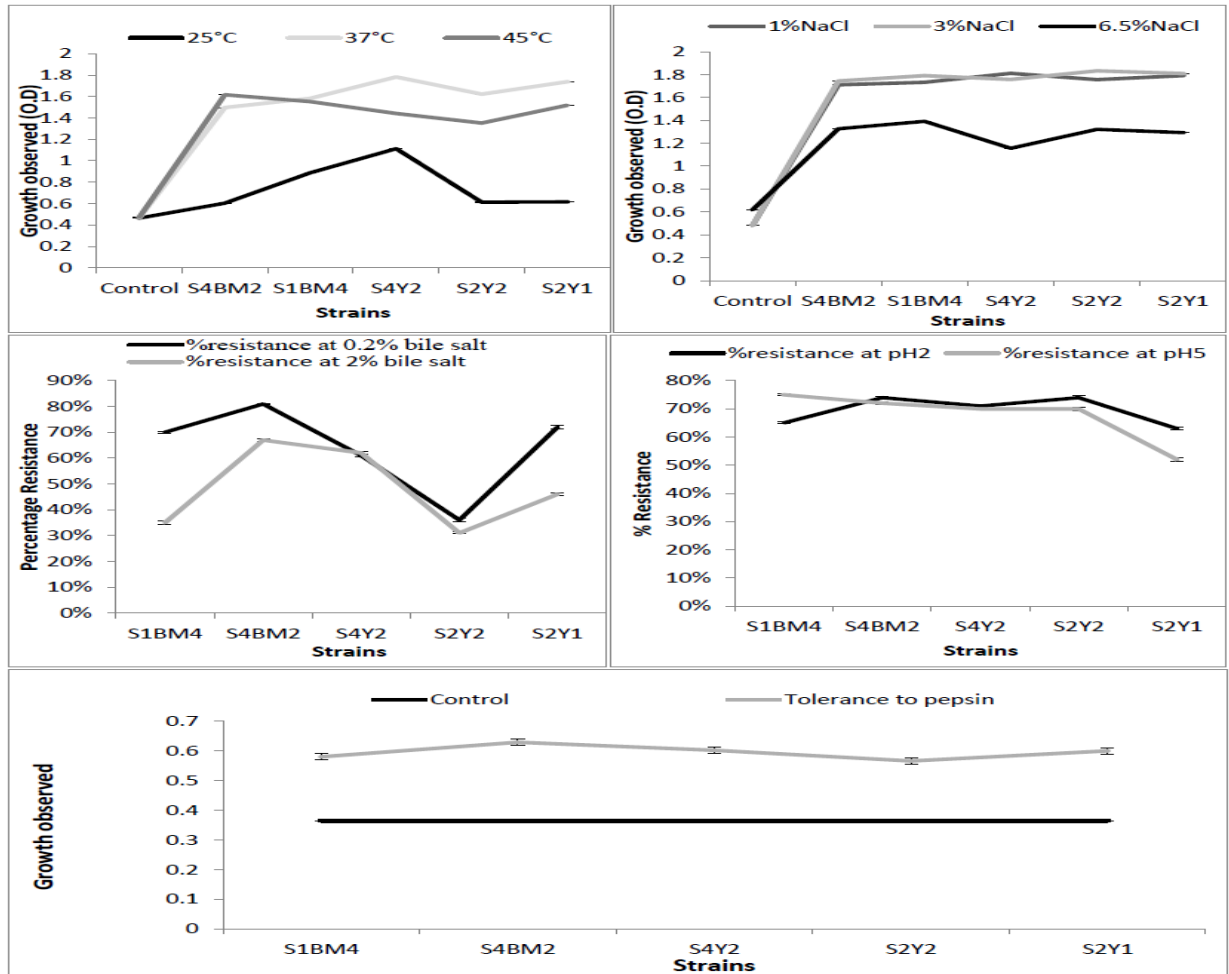


Figure 1: Identification of physiological properties and probiotic properties of Lactobacilli strains

Strains	Zone of inhibition(mm)						
	<i>Enterobacter</i> spp.	<i>E. coli</i> (9473)	<i>E. coli</i> (ETCC)	MRSA 6	<i>Klebsiella</i> spp.	<i>Salmonella typhi</i>	<i>P. aeruginosa</i>
S ₄ BM ₂	13	12	14	13	14	15	13
S ₁ BM ₄	11	11	12	12	10	14	11
S ₄ Y ₂	12	12	14	12	11	12	11
S ₂ Y ₂	11	12	11	12	10	12	11
S ₂ Y ₁	12	11	10	11	11	10	11

Table 2: Antimicrobial activity of selected Lactobacillus strains against *Enterobacter* spp., *E. coli* (9473), *E. coli* (ETCC), MRSA 6, *Klebsiella* spp., *Salmonella typhi* and *P. aeruginosa*

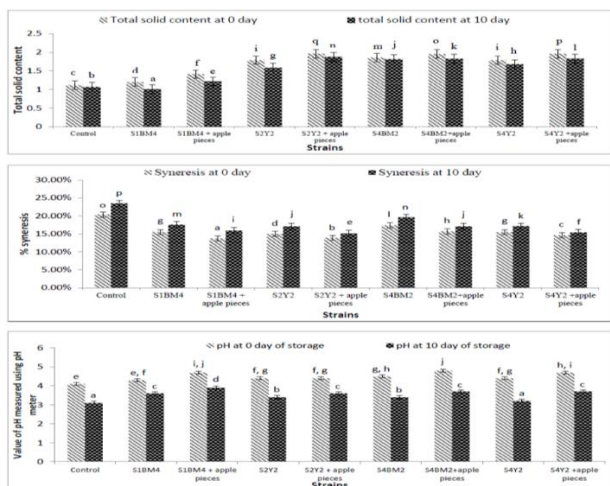


Figure 2: Syneresis, pH determination and total solid content of processed yogurt measured at 0, and 10th day of storage. Error bars represent the standard deviation of mean and different subscripts lowercase letters indicates significantly different results ($p < 0.005$).

be selected as suitable probiotic, they should be able to grow at low pH and in the presence of 0.2% of bile salt. Isolates showing $\geq 50\%$ resistance at pH 2 are considered as pH tolerant. At pH 2, 100% of the selected strains were able to resist this low pH and showed a percentage resistance of more than 50% and the greatest resistance was shown by S4BM2 (74%). However, in the study of Kumar and Kumar, highest resistance was shown by LBS2 at pH 3 which was 60.52% [18]. Isolated strains were also tested for bile salt (0.2%) tolerance. Strains showing percentage resistance $\geq 50\%$ were considered to be tolerant to bile salt (0.2%). In the screening study all of the strains were able to grow at 0.2% bile salt after 24 hours of incubation. In an earlier study, researchers had observed highest resistance of strain LBS2 with bile salt (0.3% w/v) 72.36% [18]. In our study, the maximum growth was being shown by S4BM2 which was 81%. Pepsin is an enzyme of stomach which helps in the breakdown of protein which affects the activity of these bacterial strains in acidic condition of stomach. Tested strains maintain constant viability in the presence of pepsin enzyme. Our work was correlated with Feng *et al.*, and the isolated strains were also able to grow at stimulated gastric juice [21].

Strains with good probiotic properties were selected for the production of probiotic yogurt and yogurt formed with *Lactobacillus* enriched apple pieces. Initial pH of buffalo milk was 6.5 and was decreased due to production of lactic acid by yogurt starter cultures. Yogurt sample without any strain (control) showed more decrease in pH as compared to the yogurt sample with *Lactobacillus* and the yogurt with *Lactobacillus* enriched apples at 0 day of storage. After 10th day of storage, decrease in pH of control showed more decrease in pH due to conversion of lactose into lactic acid. However, in case of yogurt produced with incorporation of enriched apple pieces, there was a delay in lactic acid production and hence less decrease in pH during the 10 day of storage [23].

The analysis of syneresis is of particular importance especially during storage. In our research, the value of syneresis at 10 day of storage varied from 23.5% to 14.9% and showed significant results as compared to yogurt processed by *Lactobacillus* strains only. Least syneresis was shown by yogurt sample processed by the addition of apple pieces enriched with S1BM4 strain. Our work was correlated with Bosnea *et al.*, [23]. In their research, less syneresis was observed in yogurt produced with enriched apple pieces as compared to control and yogurt processed with probiotic strains [23]. Total solid content of yogurt being processed by combination of *Lactobacillus* strains and apple pieces showed significant result as compared to yogurt processed by *Lactobacillus* strains and starter cultures only and the best result was shown by S2Y2 and apple pieces.

In our study we use apple pieces because they have pectin that account for total of 50% and have prebiotic benefit and when they are combined with probiotic *Lactobacillus*, they allow these strains to survive better under storage conditions. It was concluded that yogurt formed with synbiotic effect (probiotics combined prebiotics) showed better results as compared to other yogurt with only probiotic effect. This combination of apple pieces and probiotic strains can further be used in different dairy industries for yogurt processing to improve the quality of yogurt.

Conflict of Interest Statement

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contribution

Rubani U performed the experimental work and drafted manuscript. Iqbal A designed and supervised the research work, analyzed data and approved final manuscript.

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