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Microbiological analysis of synbiotic cottage cheese using *Lactobacillus acidophilus* (LA-5) and *Lactobacillus casei* (NCDC-298) with pectin

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Abstract

Cottage cheese is a fermented milk product, wherein addition of probiotics can confer a health benefit on the host. The study was conducted to estimate the probiotic viability and microbiological attributes of the synbiotic cottage cheese using *Lactobacillus acidophilus* (*LA-5*) and *Lactobacillus casei* (*NCDC-298*) with pectin. The synbiotic cottage cheese was prepared and stored at 4 °C for 14 days during which the microbiological attributes were studied on day 0, 3, 7 and 14. The highest viable probiotic count was observed for T4 (9.10 ± 0.84 $\log_{10}$ cfu/g) on day 3 of storage and the count significantly decreased to 8.50 ± 0.47 $\log_{10}$ cfu/g on day 14 of storage. The lowest count was observed for T2 (8.36 ± 0.42 $\log_{10}$ cfu/g) on day 14 storage. The highest coliform count (1.58 ± 0.40 $\log_{10}$ cfu/g) was noticed for T2 on day 3 of storage and lowest (0.69 ± 0.22 $\log_{10}$ cfu/g) was noticed for T3 on day 14 of storage. The yeast and mould count increased significantly (P<0.05) throughout the storage period.

Keywords: *Lactobacillus acidophilus, Lactobacillus casei*, pectin, probiotic viability, coliform, yeast and mould

Introduction

Cottage cheese is a dairy product which has a good potential for delivery of probiotic microorganisms into the human intestine due to its specific chemical and physical characteristics compared to fermented milks like higher pH value and lower titratable acidity, higher buffering capacity, higher nutrient availability, lower oxygen content, and denser matrix of the texture. Foods that are containing probiotic bacteria come to the forefront as having a positive effect on health. These benefits include improving the gut microbial balance, stimulation of the immune system, reduction of blood cholesterol level, and reduction in the incidence of cancer, cardiovascular diseases, diarrhea and osteoporosis (Holzapfel and Schillinger, 2002; Marteau and Boutron-Ruault, 2002; Sanders, 2003; Madureira *et al*., 2005) [6, 12, 16, 11].

Several authors have suggested that ingestion of 10^6-10^9 viable cells g-1 in the product at the moment of consumption is the minimum necessary concentration to cause a beneficial result (Gomes *et al*., 1995) [4]. *Lactobacilli* are a main component of the commensal human bacteria in the gastrointestinal tract that have been shown to be protective against pathogen infection (Hirano *et al*., 2003) [5]. *Lactobacillus* species have been successfully used in clinical trials to treat various forms of diarrhea (Mack and Lebel, 2004) [10].

Hotchkiss *et al*. (2003) [7] has proposed pectin oligosaccharide to be an excellent new generation prebiotic. He also reported that fermentation of Valencia orange peels demonstrated bifidogenic effects and the concentration of some short chain organic acid such as acetate, butyrate and propionate increased upon fermentation.

In the present situation, where gastrointestinal and other systemic diseases are most commonly occurring, probiotic or synbiotic dairy products are serving as remedy and also gaining popularity.

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Material and Method

Skim milk: Fresh whole milk was received from Livestock Farm Complex, Veterinary College and Research Institute, Namakkal, Tamil Nadu, India. The skim milk was obtained from whole milk by centrifugation method after separation of cream.

Pectin: Neotea pectin powder was purchased from Neoteric DCBA Ideas, Tamil Nadu, India and preserved in moisture proof pack for incorporation in cottage cheese.

Starter culture: Cheese starter culture, *Lactobacillus acidophilus* (*LA-5*) and *Lactobacillus casei* (*NCDC-298*) were procured in freeze dried form, from National Collection of Dairy cultures, National Dairy Research Institute, Karnal, India for the preparation of cottage cheese.

Preparation of synbiotic cottage cheese: Cottage cheese was prepared as per Blanchette *et al.* (1996) [2], with some modifications. The prebiotic pectin was added at optimum level (0.3 per cent) and the probiotics were added at 5 per cent level. In each treatment, fresh whole milk was preheated to 40 °C and the cream was separated to obtain skimmed milk. Pectin was added to the skimmed milk and it was pasteurized at 80 °C for 10 minutes then cooled to 32 °C. Calcium chloride was added at 0.02 per cent level to it and stirred well and kept for 10 minutes. Cheese culture or the probiotics were added at the rate of 5% and incubated at 37 and 30 °C, for *Lactobacillus acidophilus* (*LA-5*) and *Lactobacillus casei* (*NCDC-298*) respectively for 40 minutes to 1 hour for development of acidity. After development of acidity, rennet was added at 0.1-0.2 g per litre of milk and was incubated at 37 °C until a firm coagulum was formed. The coagulum was then cut into small cubes and cooked up to 53-56 °C for 90 minutes. The whey released during the cooking process was drained using a muslin cloth and the curd cubes were washed in water to remove excess acidity. The excess water was drained off the cubes and then it was salted at 0.6%. The developed cottage cheese was packed in polystyrene cups and stored at 4 °C.

Treatments	Description
C	Control (Skim milk + Commercial cheese culture)
T1	Skim milk + Commercial cheese culture with 0.3 per cent pectin
T2	Skim milk + *Lactobacillus acidophilus* (*LA-5*) culture with 0.3 per cent pectin
T3	Skim milk + *Lactobacillus casei* (*NCDC-298*) culture with0.3 per cent pectin
T4	Skim milk + *Lactobacillus acidophilus* (*LA-5*) + *Lactobacillus casei* (*NCDC-298*) culture with 0.3 per cent pectin

Propagation of *L. acidophilus* (*LA-5*)

The freeze-dried culture was inoculated into skimmed milk medium (11%) and incubated at 37 °C aerobically for 24-48 hours for initial propagation.

Confirmation of *L. acidophilus* (*LA-5*)

A loop full of skimmed milk medium culture was inoculated on MRS agar slant and incubated at 37 °C aerobically for 24-48 hours. After incubation, the Lactobacillus colonies were confirmed by growth, colony characteristics and gram staining as described by Kandler and Weiss (1986) [8].

Propagation of *L. casei* (*NCDC-298*)

The freeze-dried culture was inoculated into skimmed milk medium (11%) and incubated at 30 °C aerobically for 24-48 hours for initial propagation.

Confirmation of *L. casei* (*NCDC-298*)

A loop full of skimmed medium culture was inoculated on MRS (with vancomycin) agar slant and incubated at 30 °C aerobically for 24-48 hours. After incubation, the Lactobacillus colonies were confirmed by growth, colony characteristics and gram staining as described by Kandler and Weiss (1986) [8].

Gram staining

Lactobacillus acidophilus and *Lactobacillus casei* colonies were smeared on a clean glass slide with sterile normal saline. After drying, it was heat fixed and stained using Gram staining kit (Himedia, Mumbai) and viewed under oil immersion objective of microscope.

Sub-culturing of *L. acidophilus* (*LA-5*) in skimmed milk medium

Sub-culturing was done in the medium containing 11% skimmed milk powder which was reconstituted with demineralized water and autoclaved at 121 °C for 15 minutes. The medium was checked for sterility for 24 hours and then inoculated with mother culture and incubated at 37 °C aerobically until the formation of coagulum. After formation of coagulum, culture was stored at 4 °C for periodical transfer.

Sub-culturing of *L. casei* (*NCDC-298*) in skimmed milk medium

Sub-culturing was done in the medium containing 11% skimmed milk powder which was reconstituted with demineralized water and autoclaved at 121 °C for 15 minutes. The medium was checked for sterility for 24 hours and then inoculated with mother culture and incubated at 30 °C aerobically until the formation of coagulum. After formation of coagulum, culture was stored at 4 °C for periodical transfer.

Estimation of microbial parameters of cottage cheese during storage

The synbiotic cottage cheese samples intended for microbiological analysis were ground into a fine paste using a sterile pestle and mortar and subjected to the analysis.

Probiotic viability count

The probiotic viability count was determined by adding 1 ml of homogenized cheese sample into test tubes having 9 ml of sterile physiological saline solution. After thorough mixing, serial dilutions were prepared by transferring 1ml from each tube and discarding 1ml from the last dilution. Under a horizontal laminar flow cabinet, 1ml of appropriate dilutions was poured into petri plates in duplicates containing MRS agar (CDH, India) that were previously sterilized. The plated samples were allowed to solidify and then incubated at 37 ºC and 30 ºC for *Lactobacillus acidophilus* (*LA-5*) and *Lactobacillus casei* (*NCDC-298*) respectively for 24 h. Plates containing 30-300 colonies were counted using colony counter.

Coliform count
One ml of homogenized cheese sample was added into a test tube having 9 ml of sterile physiological saline solution. After thorough mixing, serial dilutions were prepared by transferring 1ml from each tube and discarding 1 ml from the last dilution. 1 ml of appropriate dilutions were poured in VRB agar plates in duplicates. The plated samples were then allowed to solidify and incubated at 37 ºC for 24 h. Plates containing 30-300 colonies were counted using colony counter.

Yeast and mould count
Yeast and mould count were made using Chloramphenicol yeast glucose agar. Plating was done as stated above for coliform count and colonies were counted after incubating the plates at 25 °C for 5 days.

Statistical analysis: The data were analyzed by two way ANOVA in SPSS (version 20.0).

Results and Discussion
Viability of probiotics
The results of this study (Table 1) revealed an increase in probiotic count from day 0 to 3 of storage for all the treatments. The highest count was observed for T4 (9.10 ± 0.84 $\log_{10}$ cfu/g) on day 3 of storage and the count significantly decreased to 8.50 ± 0.47 $\log_{10}$ cfu/g on day 14 of storage. The lowest count was observed for T2 (8.36 ± 0.42 $\log_{10}$ cfu/g) on day 14 storage. A significant difference ($P<0.05$) was noticed between day 0, 3 and 14 of storage but no significant difference was noticed between day 0 and 7. This may be attributed to multiplication of the probiotics during initial storage phase due to addition of pectin and later the reduction might be related to intensive production of lactic acid, exhaustion of nutrients and growth of yeast and moulds. These results are in accordance with Yen *et al*. (2017) [18] who obtained higher probiotic count with addition of higher concentration of pectin enzyme hydrolysate.
Similarly, Rafael *et al.* (2013) [14], developed and characterized a synbiotic cheese with *Saccharomyces boulardii* and inulin. They stated that the probiotic viability decreased during later period of storage from 0 week (9.04 $\log_{10}$ cfu/g) to 2nd week (7.30 $\log_{10}$ cfu/g) and Rebiero *et al.* (2014) [15], reported the probiotic viability of *L. acidophilus* (*LA-5*) to be 8.18, 8.16 and 8.04 $\log_{10}$ cfu/g on day 1 , 7 and 14 of storage, respectively.

Table 1: Viability of probiotics ($\log_{10}$ cfu/g) in synbiotic cottage cheese during storage at 4 °C

Treatments	Storage period (days)			
	0	3	7	14
C	8.88 ± 0.49^{Ba}	9.01 ± 0.76^{Ca}	8.91 ± 0.87^{Ba}	8.75 ± 0.49^{Aa}
T1	8.97 ± 0.61^{Bab}	9.05 ± 0.88^{Cab}	8.95 ± 0.95^{Bab}	8.79 ± 0.70^{Aab}
T2	8.98 ± 0.66^{Bb}	9.06 ±0.92^{Cb}	8.97 ± 0.92^{Bb}	8.36 ± 0.42^{Ab}
T3	8.89 ±0.47^{Ba}	9.00 ± 0.57^{Ca}	8.92 ± 0.42^{Ba}	8.39 ± 0.42^{Aa}
T4	9.02 ± 0.67^{Bc}	9.10 ± 0.84^{Cc}	9.02 ± 0.76^{Bc}	8.50 ± 0.47^{Ac}

Mean (n=6) bearing different uppercase superscripts between columns differ significantly ($P<0.05$)
Mean (n=6) bearing different lowercase superscripts between rows differ significantly ($P<0.05$)

Coliform count
The result of coliforms in synbiotic cottage cheese for different treatments during the storage are presented in Table 2. The highest count (1.58 ± 0.40 $\log_{10}$ cfu/g) was noticed for T2 on day 3 of storage and lowest (0.69 ± 0.22 $\log_{10}$ cfu/g) was noticed for T3 on day 14 of storage. The count increased significantly ($P<0.05$) from day 0 to 3 of storage and then decreased significantly on day 14 of storage, which may be due to lower pH of the cottage cheese during the initial days of storage. Currently, the PMO limits coliforms in Grade "A" pasteurized milk and milk products to 10 or fewer CFU per mL (FDA, 2015) [3]. These results are in close agreement with reports of Aylward *et al.* (1980) [1], who observed coliform count in cottage cheese to be 1.77 and 3.45 $\log_{10}$ cfu/ml on day 0 and 4, respectively.

Table 2: Coliform count ($\log_{10}$ cfu/g) of synbiotic cottage cheese during storage at 4 °C

Treatments	Storage period (days)			
	0	3	7	14
C	1.22 ± 0.42^{Babc}	1.52 ± 0.55^{Cabc}	1.26 ± 0.30^{Babc}	0.82 ± 0.33^{Aabc}
T1	1.17 ± 0.42^{Bab}	1.33 ± 0.60^{Cab}	1.12 ± 0.33^{Bab}	0.81 ± 0.21^{Aab}
T2	1.39 ± 0.42^{Bbc}	1.58 ± 0.40^{Cbc}	1.26 ± 0.30^{Bbc}	0.70 ± 0.22^{Abc}
T3	1.00 ± 0.51^{Ba}	1.36 ± 0.84^{Ca}	1.17 ± 0.86^{Ba}	0.69 ± 0.22^{Aa}
T4	1.33 ± 0.30^{Bc}	1.57 ± 0.40^{Cc}	1.42 ± 0.33^{Bc}	0.92 ± 0.16^{Ac}

Mean (n=6) bearing different uppercase superscripts between columns differ significantly ($P<0.05$)
Mean (n=6) bearing different lowercase superscripts between rows differ significantly ($P<0.05$)

Yeast and mould count
The results for yeast and mould in synbiotic cottage cheese for different treatments during the storage are presented in Table 3. The yeast and mould count significantly ($P<0.05$) increased from day 0 to 14 of storage. The lowest count was observed for T4 (1.07 ± 0.31 $\log_{10}$ cfu/g) on day 0 of storage and it increased up to 1.95 ± 0.31 $\log_{10}$ cfu/g on day 14. The highest count was observed for T3 (1.26 ± 0.31 $\log_{10}$ cfu/g) on day 0 of storage and it increased to 2.06 ± 0.62 $\log_{10}$ cfu/g on day 14 of storage. This may be attributed to favorable low pH of the product for the growth of yeast and moulds during storage period. However, the count was within the acceptable range of less than 10 as reported by USDA, (2001) [17]. This study is in accordance with findings of Pereira-Dias *et al.* (2000) [13], they observed the mean yeast count of artisan Portuguese ewe's cheese to range from 2.7 to 6.4 $\log_{10}$ cfu/g. Similarly, Karen and Bouma (1992) [9], observed the yeast and mould count to remain within a log cycle throughout the shelf life of cottage cheese.

Table 3: Yeast and mould count (log_{10} cfu/g) of synbiotic cottage cheese during storage at 4 °C

Treatments	Storage period (days)			
	0	3	7	14
C	1.22 ± 0.33^{Ac}	1.65 ± 0.43^{Bc}	1.92 ± 0.49^{Cc}	2.17 ± 0.49^{Dc}
T1	1.12 ± 0.33^{Ab}	1.52 ± 0.33^{Bb}	1.86 ± 0.54^{Cb}	2.07 ± 0.54^{Db}
T2	1.22 ± 0.33^{Ab}	1.64 ± 0.61^{Bb}	1.87 ± 0.84^{Cb}	2.06 ± 0.99^{Db}
T3	1.26 ± 0.31^{Ab}	1.67 ± 0.49^{Bb}	1.89 ± 0.48^{Cb}	2.06 ± 0.62^{Db}
T4	1.07 ± 0.31^{Aa}	1.50 ± 0.48^{Ba}	1.78 ± 0.37^{Ca}	1.95 ± 0.31^{Da}

Mean (n=6) bearing different uppercase superscripts between columns differ significantly ($P<0.05$)
Mean (n=6) bearing different lowercase superscripts between rows differ significantly ($P<0.05$)

Conclusion

In the present study, it was observed that the synbiotic cottage cheese prepared using combination of prebiotic pectin and probiotics *L. acidophilus* (*LA-5*) and *L. casei* (*NCDC-298*), had better results for probiotic viability, yeast and mould count than the control and coliform count within the prescribed limit.

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