

Development of healthy whey drink with *Lactobacillus rhamnosus*, *Bifidobacterium bifidum* and *Propionibacterium freudenreichii* subsp. *shermanii*

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Summary

Whey beverage was prepared by utilizing Lactobacillus rhamnosus NCDO 243, Bifidobacterium bifidum NCDO 2715 and Propionibacterium freudenreichii subsp. shermanii MTCC 1371 in order to make a fermented probiotic healthy drink. The product made with 4 % mixed culture (1:1:1) inoculated (initial count - lactobacilli 6.2×10^7 CFU/mL, bifidobacteria 5.4×10^7 CFU/mL, propionibacteria 3.9×10^7 CFU/mL) in deprotienized whey (4.6 % lactose, 0.62 % ash, 0.48 % fat and 0.5 % protein) adjusted to pH 6.4 and incubated at 37 °C for 8 h has a good technological and dietetic criteria required for a probiotic product. Total bacterial count, lactobacilli count, bifidobacteria count, propionibacteria count, titratable acidity, β -D galactosidase activity, concentration of lactic acid and sensory properties were monitored during storage period. The whey beverage fermented for 8 h and prepared with 4 % inoculum of mixed culture (1:1:1) met the probiotic criterion by maintaining each type of bacterial population at counts greater than 10^8 CFU/mL up to 10 days of storage period. The titratable acidity as well as sensory properties did not change appreciably during first 7 days of storage. At the end of 15 days of storage, slight acidification was detected, although the beverage still retained an acceptable flavour.

Key words: probiotic, whey, fermented beverage, antibacterial activity

Introduction

Probiotic lactic acid bacteria have been studied as dietary sources of live micro-organisms destined to promote a positive impact in the host by improving the properties of the indigenous beneficial microbiota (Klaenhammer et al., 1998). Regular intake of fermented dairy products look effective in treatment of various illness viz. gastrointestinal disorders (Gurr, 1984), hypercholesterolemia (Deeth and Tamine, 1981) antitumoral

(Ayebo et al., 1981) reduction of protein allergies, treatment of vaginal discharge, a cure for osteoporosis (Deeth and Tamine, 1981), improvement of vitamin synthesis and calcium absorption (Vinderola et al., 2000), development of longer villi and significantly deeper crypts in the ideal region of the gut and production of substances of low molecular mass with antimicrobial activity (Axeleson et al., 1989; Cases et al., 1998). It is therefore understandable that there has been an increasing interest in the incorporation of the species into the fermented dairy products. Traditionally, probiotics have been added to yoghurt, and it is estimated that currently more than 80 products containing lactobacilli or bifidobacteria are being produced worldwide, including sour cream, buttermilk and frozen desserts. Recently the key growth sector has been probiotic drinks (Shah et al., 1995). *Bifidobacterium bifidum* and in very recent years *Lactobacillus rhamnosus* have been considered important probiotic microorganisms for their good therapeutic properties. Conway et al., (1987) reported that *Lactobacillus rhamnosus GG* tolerate the high acid concentration in the stomach and bile acid in the intestine. Acting as a probiotic *Lactobacillus rhamnosus GG* is claimed to colonize in the digestive tract and balance the intestinal microflora. *Lactobacillus rhamnosus GG* was used successfully for the first time to treat gastrointestinal carriage of vancomycin-resistant enterococci (VER) in renal patients (Manley et al., 2007). On the other hand *Propionibacterium freudenreichii* subsp. *shermanii* is reported to synthesize various vitamins and to possess lactase activity and antibacterial properties which leads to possibility of their usage as a dietary supplements. (Yongzhe et al., 2004). Therefore there has been an increasing interest in incorporation of these species into fermented dairy products.

The present investigation includes a report on the preparation of fermented whey beverage prepared by using *Lactobacillus rhamnosus*, *Bifidobacterium bifidum* and *Propionibacterium freudenreichii* subsp. *shermanii* as culture microorganisms and assessment of its acceptability containing a population of *Lactobacillus rhamnosus*, *Bifidobacterium bifidum* and *Propionibacterium freudenreichii* subsp. *shermanii* greater than 10^8 CFU/mL during storage with good dietetic characteristics.

Materials and methods

Maintenance of probiotic strains

Bifidobacterium bifidum NCDO 2715 and *Lactobacillus rhamnosus* NCDO 243 (obtained from National Collection of Dairy Organisms, National Dairy Research Institute, Karnal, India) and *Propionibacterium freudenreichii* subsp. *shermanii* MTCC 1371 (obtained from the Institute of Microbial Technology, Chandigarh, India) were maintained in sterile skim milk fortified with 1 % dextrose and 0.1 % yeast extract. The stock cultures were activated by three successive transfers at 24 h interval.

The virulent pathogenic strains of *Bacillus cereus*, *Escherichia coli*, *Shigella dysenteriae* and *Staphylococcus aureus* were obtained from Department of Veterinary Microbiology, WBUAFS, and Kolkata, India and were maintained on nutrient agar slants by weekly propagation and activated by three successive transfers at 24 h interval in nutrient broth.

Preparation of whey

Whey was prepared by heating cow milk to 82 °C and 2 % citric acid solution was added at the rate of 2 g per kg of milk. Complete coagulation was

Table 1: Effect of inoculum level on acidity, total cell count and antibacterial activity of whey drink

Tablica 1: Utjecaj stupnja inokulacije na kiselost, ukupni broj bakterija i antibakterijsku aktivnost napitka sirutke

Percent inoculum Postotak inokuluma	Acidity Kiseelost (LA %)	Total cell count Ukupni broj bakterija (CFU/mL)	Dia. of zone of inhibition (mm)* Promjer zone inhibicije (mm)*			
			<i>E. coli</i>	<i>S. aureus</i>	<i>Shigella dysenteriae</i>	<i>B. cereus</i>
1	0.62	3.93x10 ⁷	9.0	9.0	9.0	8.0
2	0.70	4.72x10 ⁷	9.5	9.5	9.5	8.0
4	0.78	11.7x10 ⁸	10.5	11.0	10.5	8.5
8	0.92	18.58x10 ⁸	9.0	8.0	7.5	7.5

Culture combination: *Lactobacillus rhamnosus* NCDO 243, *Bifidobacterium bifidum* NCDO 2715 and *Propionibacterium freudenreichii* subsp. *shermanii* MTCC 1371 (1:1:1 at 37 °C for 8 h)

Kombinacija kultura: *Lactobacillus rhamnosus* NCDO 243, *Bifidobacterium bifidum* NCDO 2715 i *Propionibacterium freudenreichii* subsp. *shermanii* MTCC 1371 (1:1:1 na 37 °C, 8 h)

* Well diameter included (5 mm)/Uključivši promjer udubine za uzorak (5 mm)

- No inhibition observed/Nema inhibicije

effected within one minute and the whey filtered through muslin cloth is popularly known as *chhana whey* where the coagulum is used as a base material for the preparation of traditional sweetmeats in India. Whey obtained was adjusted to pH 5.5 using 10 % NaHCO₃ solution and was heated at 100 °C for 10 min with 0.4 % CaCl₂ and kept undisturbed overnight at room temperature and filtered to obtain deproteinized whey (Mathur et al., 1986). The average composition of whey was 4.6 % lactose, 0.62 % ash, 0.48 % fat and 0.5 % protein.

Preparation of healthy whey drink

Whey was enriched by 8 % sucrose (Qualigence fine chemicals, Mumbai, India) and pasteurized at 80 °C for 30 min. and cooled to 37 °C. Then it was inoculated with probiotic culture of *Lactobacillus rhamnosus* NCDO 243, *Bifidobacterium bifidum* NCDO 2715 and *Propionibacterium freudenreichii* subsp. *shermanii* MTCC 1371 (1:1:1) at the rate of 4 % and incubated at 37 °C. When the maximum bacterial population reached (8 h), fermentation was stopped by quick chilling.

Analysis

Chemical analysis

Whey drink was analyzed for titratable acidity (BIS, 1960), concentration of lactic acid (Baker and Summerson, 1941), β-D galactosidase activity (Citti et al., 1965) and volatile acidity (Hempeniens and Liska, 1968).

Microbiological analysis

The viable count of probiotic bacteria was determined using plate count method (Vinderola and Bailo, 2000) and the result were expressed as CFU /mL. All plates were incubated aerobically at 37 °C for 48 h. The product was analyzed for lactobacilli count using Modified MRS agar (Hull and Robert, 1984), propionibacteria count using sodium lactate agar (Hettinga et. al., 1968) and bifidobacteria count using Yoshioka agar (Yoshioka et al., 1968). The antibacterial activity of the product was estimated by modified cup agar assay technique (BSI, 1968). Culture filtrate (cell free extract) was collected by centrifugation at 3000 r.p.m. for 15-20 min. These were passed through Seitz filter separately. Wells of 5 mm diameter were made on solidified nutrient agar (inoculated with pathogenic test organism) in each plate and 50

μL were transferred to the wells. The plates were incubated without inverting at 37 °C for 18-24 h and diameters of inhibition zones were statistically evaluated by analysis of variance (Snedecor and Cochran, 1967).

Sensory evaluation

Sample of whey beverage were subjected to sensory evaluation by a panel of 7 judges on 9 point hedonic scale (Amerine, 1967) and analyzed statistically by two way classification (Snedecor and Cochran, 1967).

Results and discussions

Effect of inoculum level

The effect of 1, 2, 4, and 8 % inoculum of *Lactobacillus rhamnosus* NCDO 243, *Bifidobacterium bifidum* NCDO 2715 and *Propionibacterium freudenreichii* subsp. *shermanii* MTCC 1371 (1:1:1) on titratable acidity, total count and antibacterial activity is depicted in table 1. Enhanced titratable acidity (0.62-0.92 %) and total count (3.93×10^7 - 18.58×10^8 CFU/mL) were observed with the increased level of inoculum. A 4 % level of inoculum

Table 2: Effect of incubation temperature on acidity, total cell count and antibacterial activity of whey drink

Tablica 2: Utjecaj temperature inkubacije na kiselost, ukupni broj bakterija i antibakterijsku aktivnost napitka sirutke

Incubation temperature Temperatura inkubacije (0 °C)	Acidity Kiselost (LA %)	Total cell count Ukupni broj bakterija (CFU/mL)	Dia. of zone of inhibition (mm)* Promjer zone inhibicije (mm)*			
			<i>E. coli</i>	<i>S. aureus</i>	<i>Shigella dysenteriae</i>	<i>B. cereus</i>
37	0.78	11.7×10^8	10.5	11.0	10.5	8.5
42	0.79	10.5×10^8	9.0	9.5	9.0	-
45	0.83	9.6×10^8	7.0	7.5	7.0	-

Culture combination: *Lactobacillus rhamnosus* NCDO 243, *Bifidobacterium bifidum* NCDO 2715 and *Propionibacterium freudenreichii* subsp. *shermanii* MTCC 1371 (1:1:1 at 4% level for 8 h)

Kombinacija kultura: *Lactobacillus rhamnosus* NCDO 243, *Bifidobacterium bifidum* NCDO 2715 i *Propionibacterium freudenreichii* subsp. *shermanii* MTCC 1371 (1:1:1 s 4 %, 8 h)

*Included well diameter of well (5mm) (amount of supernatant in well. 0.05 mL)/Uključivši promjer udubine za uzorak (5mm) (količina uzorka u udubini 0,05 mL)

showed maximum antibacterial activity against *Escherichia coli* (10.5 mm), *Staphylococcus aureus* (11 mm), *Shigella dysenteriae* (10.5 mm) and *Bacillus cereus* (9.5 mm); however the antibacterial activity against these microorganisms decreased at inoculum level of 8 % (9 mm, 8 mm, 7.5 mm and 7.5 mm respectively). Although highest titratable acidity (0.92 %) and total count (18.58×10^8 CFU/mL) were observed at 8% level of inoculum, but 4 % level of inoculum was significantly better considering the antibacterial activity as a major criteria.

Effect of incubation temperature

The data on the effect of various incubation temperature viz. 37 °C, 42 °C, 45 °C on acidity (% LA), total count and antibacterial activity of the whey drink are presented in table 2. The maximum titratable acidity (0.83 %) was observed at the incubation temperature of 45 °C followed by 42 °C (0.79 %) and 37 °C (0.78 %) respectively. However total viable count was found maximum at 37 °C (11.7×10^8 CFU/mL). Weak inhibition zone against

Table 3: Effect of concentration of sugar on acidity, total cell count and antibacterial activity of whey drink

Tablica 3: Utjecaj koncentracije šećera na kiselost, ukupni broj bakterija i antibakterijsku aktivnost napitka sirutke

Concentration of sugar, % Koncentracija šećera, %	Acidity Kiselost (LA %)	Total cell count Ukupni broj bakterija (CFU/mL)	Dia. of zone of inhibition (mm)* Promjer zone inhibicije (mm)*			
			<i>E. coli</i>	<i>S. aureus</i>	<i>Shigella dysenteriae</i>	<i>B. cereus</i>
0	0.78	11.7×10^8	9.5	10.5	9.5	8.5
6	0.78	11.7×10^8	9.5	10.5	9.5	8.5
8	0.78	11.7×10^8	10.5	11.0	10.5	8.5
10	0.79	11.4×10^8	9.5	10.0	9.5	8.0
12	0.72	10.8×10^8	8.5	9.5	8.5	7.0
16	0.63	3.6×10^8	-	-	-	-

Culture combination: *Lactobacillus rhamnosus* NCDO 243, *Bifidobacterium bifidum* NCDO 2715 and *Propionibacterium freudenreichii* subsp. *shermanii* MTCC 1371 (1:1:1 at 4 % level, incubated at 37 °C for 8 h)

Kombinacija kultura: *Lactobacillus rhamnosus* NCDO 243, *Bifidobacterium bifidum* NCDO 2715 i *Propionibacterium freudenreichii* subsp. *shermanii* MTCC 1371 (1:1:1 s 4 %, inkubacija na 37 °C, 8 h)

*Well diameter included (5 mm)/Uključivši promjer udubine za uzorak (5 mm)

- No inhibition observed/Nema inhibicije

Escherichia coli (9 mm and 7.0 mm), *Shigella dysenteriae* (9.5 mm and 7.5 mm) and *Staphylococcus aureus* (7 mm and 9 mm) were observed or no antibacterial activity against *Bacillus cereus* were visible at the incubation temperature of 42 °C and 45 °C respectively. The data indicated that production of antibacterial substances is not related to the titratable acidity. Similar results were also reported by Chopra and Gandhi (1989). This may be due to the increased cell count of 11.7×10^8 CFU/mL promoted by the incubation temperature of 37 °C that leads to the production of antibacterial substances.

Effect of sugar concentration

Effect of different concentration of sucrose (0, 6, 8, 10, 12, and 16 %) on titratable acidity, total count and antibacterial activity are presented in table 3. Slight change in titratable activity (0.78 - 0.79 %), total count (11.7×10^8 - 11.4×10^8 CFU/mL) and antibacterial activity (9.5 mm - 10.5 mm against the pathogenic test organisms) were observed up to 10 % sugar concentration, but at 12 % sucrose level the changes were significant ($p < 0.05$). Addition of 16 % sugar exhibited no antibacterial activity against any of the four test organisms with low acidity (0.63 %) and viable count (3.60×10^8 CFU/mL). Tramer (1973) reported that during preparation of yoghurt, addition of sugar should not allow total solid to exceed 22 % to avoid the severe inhibition of yoghurt starters. However it was observed that whey drink with 8 % level of sucrose was excellent in taste with optimum titratable acidity (0.78 %) and recommended viable count of 11.7×10^8 CFU/mL.

Changes of technological and dietetic characteristics at refrigeration temperature

The final product had a titratable acidity of 0.78 %, 4.6 % lactose, 0.62 % ash, 0.48 % fat and 0.5 % protein 2.6 mL volatile acidity, 215 µg/mL lactic acid, β-d galactosidase activity 56 µg ONP/mL, mild acidic flavour and showed antibacterial activity against all the four test organisms viz, *Escherichia coli*, *Shigella dysenteriae*, *Staphylococcus aureus* and *Bacillus cereus* (inhibitory zone 10.5 mm, 11 mm, 10.5 mm and 8.5 mm respectively). Significant decline ($p < 0.05$) in β-d galactosidase activity in whey drink during 15 days of storage at 5 ± 1 °C (Table 4) may be attributed to the increase in titratable acidity or to the decrease to pH as suggested by Kirala and Shahani (1996). No change in lactic acid content (215 µg/mL) of whey drink during 15 days of storage may be due to an equilibrium between the increase in lactic

Table 4: Changes in technological and dietetic characteristics of whey beverage milk during storage at 5 ± 1 °C

Tablica 4: Promjene u tehnološkim i dijetetskim svojstvima napitka sirutke tijekom skladištenja na 5 ± 1 °C

Characteristics Svojstva	Days of storage Dani pohrane						
	0	1	3	5	7	10	15
Titrateable acidity (% lactic acid) Titracijska kiselost (% mliječne kiseline)	0.78	0.78	0.79	0.83	0.89	0.96	1.04
Lactic acid Mliječna kiselina ($\mu\text{g/mL}$)	215	215	215	215	215	215	215
β -D-galactosidase activity β -D-galaktozidazna aktivnost ($\mu\text{g ONP/mL}$)	56	45	32	26	23	19	16
Volatile acidity Hlapiva kiselost (mL 0.1N NaOH/50g of curd)	2.3	2.4	2.5	2.6	2.6	2.7	2.7
Total cell count (CFU/mL) Ukupni broj bakterija (CFU/mL)	11.7×10^8	11.7×10^8	11.2×10^8	10.7×10^8	10.2×10^8	9.5×10^8	36.7×10^7
Propionibacteria count $\times 10^8$ Broj propionibakterija $\times 10^8$ (CFU/mL)	2.9×10^8	2.9×10^8	2.7×10^8	2.5×10^8	2.3×10^8	2.1×10^8	9.3×10^7
Lactobacilli count $\times 10^8$ Broj laktobacila $\times 10^8$ (CFU/mL)	5.3×10^8	5.3×10^8	5.1×10^8	5.0×10^8	4.8×10^8	4.4×10^8	18.8×10^7
Bifidobacteria count $\times 10^8$ Broj bifidobakterija $\times 10^8$ (CFU/mL)	3.6×10^8	3.6×10^8	3.2×10^8	3.1×10^8	3.0×10^8	2.8×10^8	9.7×10^7
Sensory score Senzorna ocjena	7.3	7.3	7.1	7.0	6.90	6.30	5.2

Culture combination: *Lactobacillus rhamnosus*, *Bifidobacterium bifidum* and *Propionibacterium freudenreichii* subsp. *shermanii* (1:1:1 at 4 % level, incubated at 37 °C for 8 h)

Kombinacija kultura: *Lactobacillus rhamnosus*, *Bifidobacterium bifidum* i *Propionibacterium freudenreichii* subsp. *shermanii* (1:1:1 s 4 %, inkubacija na 37 °C, 8 h)

*Well diameter included (5 mm)/Uključivši promjer udubine za uzorak

acid production by *Lactobacillus rhamnosus* NCDO 243, *Bifidobacterium bifidum* NCDO 2715, and its utilization by *Propionibacterium freudenreichii* subsp. *shermanii* MTCC 1371 as suggested by Paker and Moon (1982). No significant variation ($p > 0.05$) in the viable count of propionibacteria, lactobacilli and bifidobacteria (Table 4) was observed during 7 days of storage. The total bacterial count decreased from 11.7×10^8 to 36.7×10^7 CFU/mL after 15 days of storage. The product was slightly sour in taste at the end of 15 days of storage and was not preferred by the consumers. However

the product was acceptable on the basis of mouth feel, overall appearance and optimum level of acidity up to 7 day of storage (sensory score 6.90).

Conclusion

The fermented whey beverage met the probiotic criteria by maintaining both bacterial population ($>10^8$ CFU/mL), therapeutic properties and optimum sensory qualities with a shelf life of 5 days. The final product preserved an acceptable flavour. The result of this research suggests that this whey beverage may be attractive for entering the growing market of probiotic.

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RAZVOJ ZDRAVOG NAPITKA SIRUTKE SA LACTOBACILLUS RHAMNOSUS, BIFIDOBACTERIUM BIFIDUM I PROPIONIBACTERIUM FREUDENREICHII SUBSP. SHERMANII

Sažetak

Napitak sirutke je pripremljen korištenjem *Lactobacillus rhamnosus* NCDO 243, *Bifidobacterium bifidum* NCDO 2715 i *Propionibacterium freudenreichii* subsp. *shermanii* MTCC 1371 u svrhu pripravljanja probiotskog zdravog napitka. Proizvod je pripremljen s 4 % inokuluma mješovite kulture (1:1:1, početni broj - laktobacili $6,2 \times 10^7$ CFU/mL, bifidobacteria $5,4 \times 10^7$ CFU/mL, propionibacteria $3,9 \times 10^7$ CFU/mL) u deproteiniziranoj sirutki (4,6 % laktoze, 0,62 % pepela, 0,48 % masti i 0,5 % proteina) prilagođenog pH (6,4) i inkubiranog na 37 °C kroz 8 h radi postizanja dobrih tehnoloških i dijetetskih kriterija za dobivanje probiotičkog proizvoda. Tijekom skladištenja promatrani su ukupni broj bakterija, broj laktobacila, broj bifidobakterija, koncentracija mliječne kiseline te senzorska svojstva. Sirutka fermentirana s 4 % inokuluma mješovite kulture tijekom 8 sati postigla je kriterije zadane za probiotičke napitke, s brojem svakog pojedinog soja većim od 10^8 CFU/mL tijekom 10 dana skladištenja. Titracijska kiselost i senzorska svojstva nisu se značajno mijenjala tijekom 7

dana skladištenja. Na kraju 15-tog dana skladištenja, pojavila se blaga kiselost iako je napitak još uvijek bio ocijenjen kao prihvatljiv obzirom na okus.

Ključne riječi: probiotik, sirutka, napitak, antibakterijska aktivnost

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