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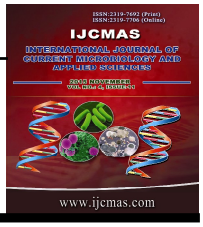

Original Research Article

Shelf Life Expanding of Fermented Dairy Food by Use of Cold Sensitive Mutant Starters

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A B S T R A C T

The use of cold-sensitive (CS) starters can serve as an alternative solution for expanding shelf life of fermented dairy products. Five CS mutants of *Lactobacillus acidophilus* MDC 9626 losing ability to grow on LAPTg and ferment milk at minimal temperature were obtain among 500 rifampicin-resistant (Rif) mutants by use of replica plating technique. All CS mutants mainly retain growth and milk coagulation rate when grow at optimal temperature, as well as the organoleptic properties of fermented dairy product. Even one of the CS Rif mutants (CS-Rif6) also possesses high growth and milk fermentation rates. Dairy preparations fermented by CS starters retain the viable cells count, titratable acidity, texture and flavor at last for four week of cold storage (in contrast to the native starter). Thus, conditionally lethal CS *rif* mutations can be used for expanding of the shelf life of dairy products at fridge temperatures as well as for improving technological characteristics of starter cultures.

Introduction

Metabolic activity of dairy starters at low temperatures during cooling and storage cause shelf life reducing of dairy products. Currently for expanding shelf life of dairy products are used pasteurization or chemical preservatives, which affect the viability of useful probiotic microorganisms and refused from consumers (Tamime and Robinson, 2011). The microorganisms growth (especially in rich media) at extreme temperatures limited by cold or heat sensitivity of one or more key protein(s) involved in global cellular processes such as DNA replication, transcription, translation or cell division (Lengeler *et al.*, 1999). There are fundamental connections between rifampicin resistance, RNA polymerase structure and function and global gene expression. Rifampicin can specifically block transcription initiation but not elongation (Ingham and Furneaux, 2000). Resistance to rifampicin is associated with mutations in the gene coding for the beta subunit of RNA polymerase (*rpoB*) (Garibyan *et al.*, 2003; Lai *et al.*, 2002). *Rif* mutations of RNA polymerase have been found involved in a variety of physiological processes and possessing pleiotropic effects,

including: cell growth (Jin and Gros, 1989; Kogoma, 1994; Maughan *et al.,* 2004); the ability of mutants to support the growth of various bacteriophages; the ability to maintain the F' episome; interaction with other genes mutant alleles; uracil sensitivity; exopolysaccharides over synthesis and thermosensitivity of LABs (Hovhannisyan *et al.,* 2010; Hovhannisyan and Barseghyan, 2015). A spontaneous *rif* mutation of *Saccharopolys poraerythraea* caused slow-growth and stimulated erythromycin production by global changing of transcriptional profile (Elisabetta *et al.,* 2009).

We suppose that the conditionally lethal CS mutations of RNA polymerase by shift up of T_{min} will limit the metabolic activity of LAB starters at low temperatures. The aim of this work is to select rifampicin resistance CS starters and evaluate the shelf life of fermented dairy products.

Materials and Methods

The strain of *Lactobacillus acidophilus* MDC9626 obtained from the Microbial Depository Center of NAS RA.

Skimmed milk, LAPTg medium (0.5% bactotryptone, 0.3% yeast extract, 0.1% glucose, Tween 80), for softening 1.5% agar was added (Huys *et al.,* 2002).

N-methyl-N′-nitro-N-nitrosoguanidine (NTG) induced mutagenesis was performed as described earlier (Hovhannisyan *et al.,* 2010). Rifampicin and NTG purchased from Serva (Germany).

NTG mutagenesis and selection of rifampicin-resistant CS mutants

Rifampicin-resistant mutants were obtained from 10^9 CFU/ml of *L. acidophilus* MDC 9626, treated by 300 μ/ml NTGby plating on LAPTg agar containing 100 μg/ml of rifampicin and incubated at 37°C.

CS mutants screened by replica plating of Rif colonies and cultivating at 23°C. Colonies, which were not growing at minimal temperature, were isolated for farther investigation.

Growth rate

Overnight cultures diluted 20 times in fresh LAPTg broth and growth at 37°C with shaking at 200 rpm. OD_{600} measurements made every 30 min.

Milk coagulation rate

1.8 ml of sterile skim milk inoculated by 0.2 ml of overnight broth cultures and incubated at 37°C and checked clotting by 30 min intervals.

Titratable acidity

The titratable acidity of the fermented milk was performed according to Thorner (^oT) (Tamime and Robinson, 2011).

Shelf life test

The 50 ml aliquots of pasteurized skimmed milk inoculated by mutant starter were poured in 12 properly covering cans, fermented for 6 hours at 37°C, and stored in refrigerator at 6°C. Three cans of milk fermented by each starter tested on physical, microbiological and chemical characteristics at a week intervals.

Statistical analysis

Statistical analysis of the data was performed using Student's t test computer, taking the criterion of $P < 0.05$ sufficient for significant differences in the results.

Results and Discussion

Phenotypic character of *L. acidophilus* MDC 9626

The strain *L. acidophilus* MDC 9626 isolated from human origin has temperature growth optimum (T_{opt}) 37–42 °C, minimum (T_{min}) 20°C and maximum (T_{max}) 48°C, cells are non-motile rods, gram-positive, catalase negative, H_2O_2 producing, oxidase positive.In contrast to many *L. acidophilus* strains, which purely grow in the milk, this strain to acidify milk by high rate and forms a solid clot.

Obtaining of rifampicin resistant mutants

Because of at least 95% of isolated rifampicin-resistant mutations clustered in a very narrow 81-bp region in *rpoB* gene (Telenti *et al.,* 1993), their spontaneous frequency is very low (3.5×10^{-8}) (Hua *et al.,* 2010). Earlier we have demonstrated that use of NTG induced mutagenesis increases the yield of *rif* mutations in LAB up to2.2×10^{-5} (Hovhannisyan *et al.,* 2010). In this study, using of NTG mutagenesis allowed increasing the frequency yield of rifampicin resistant mutants of *L. acidophilus* MDC 9626 from 4.1×10^{-8} to 3.3×10^{-5}. About 500 colonies formed on LAPTg agar with 100μg/ml of rifampicin were replica plated on the same medium and incubated at 23°C. The colonies, which are not growing on replica plates, were picked from mother plates for further investigation. It was estimated that among the Rif mutants the frequency of obtaining CS strains about 1.0%. Five of Rif mutants, which constantly have lost the ability to grow at minimal temperature suggested as CS.

The temperature range of *L. acidophilus* MDC 9626 CS mutants growth in LAPTg broth was investigated (Table 1). As seen from table 1, due to *rif* mutations minimal temperatures of growth of CS mutants increased from 4 to 9 degrees, but the optimal temperatures were not changed (37–42°C).

The growth rate of CS mutants in LAPTg medium at 37°C was determined (Figure 1).

The curves presented in figure 1, show that most of CS mutants by growth rate at optimal temperature are not significantly differ from parental strain. Except of CS-Rif 6 mutant, which growth rate was higher, and CS-Rif 9, which growth rate was lower, than that of parental strain.

The comparative rate of milk coagulation by CS starters at 37°C was studied (Figure 2).

As can be seen from figure 2, the highest milk coagulation rate (4.5 h) has mutant CS-Rif 6, the others are not differ from the parental strain. The mutants CS-Rif 7 and CS-Rif 9 coagulated milk slowly for 5.5 and 6.0 h respectively.

Milk fermentation profile by mutant starters in temperature range from 20 to 48 °C were studied (Table 2).

As seen from table 2, CS-Rif 4, CS-Rif 3, CS-Rif 9 mutants lost the ability to ferment milk below 30°C. For others the minimum temperature of milk curdling is 24°C. One of the mutants did not ferment milk at 48°C.Thus, the temperature minimum of milk fermentation of CS mutants increased on 4–9°C. It has considered that quality of dairy probiotic products estimated by the number of viable microorganisms and organoleptic properties (Shafiee *et al.,* 2010, Beal *et al.,* 1999). In connection with this has been studied the microbial titer and titratable acidity of yogurts during storage at 6°C. As criteria for estimation of product quality fermented by CS starters were used

titer of viable cells (CFU/ml) and organic acids quantity (titratable acidity, ˚T) (Table 3).

It was revealed that after first day of storage the titer of cells and titratable acidity of fermented by *L. acidophilus* MDC 9626 milk were higher, than in that in the products where used CS mutants starters, because of the parental strain continue to grow at lower temperature, when the growth of CS mutants already stopped. Whereas at further storage the viability of CS mutants stay higher and products acidity are lower than for *L. acidophilus* MDC 9626. These data suggest the long preservation of dairy products quality, fermented by use of CS mutants.

Table.1 Definition of the radical growth temperatures of CS mutants in LAPTg broth

Strains	Radical and optimal growth temperatures of CS mutants, °C		
	T_{min}	T_{opt}	T_{max}
MDC 9626	20	37 - 42	48
CS-Rif 4	28	37 - 42	48
CS-Rif 6	24	37 - 42	48
CS-Rif 3	29	37 - 42	48
CS-Rif 7	24	37 - 42	45
CS-Rif 9	29	37 - 42	45

Table.2 Temperature profile of milk fermentation by CS mutant starters

Strains	Temperature, °C							
	20	24	27	30	37	42	45	48
MDC9626	+	+	+	+	+	+	+	+
CS-Rif 4	-	-	-	+	+	+	+	+
CS-Rif 6	-	+	+	+	+	+	+	+
CS-Rif 3	-	-	-	+	+	+	+	+
CS-Rif 7	-	+	+	+	+	+	+	-
CS-Rif 9	-	-	-	+	+	+	+	+

"+" – ferment, "-" - not ferment

Table.3 Number of viable cells and titratable acidity of milk fermented by CS mutant starters of *L. acidophilus* MDC 9626 during storage at 6 °C

Strains	Days of storage							
	1		7		14		28	
	CFU/ml	˚T	CFU/ml	˚T	CFU/ml	˚T	CFU/ml	˚T
MDC 9626	2.1×10^9	80	1.2×10^9	82	6.4×10^8	100	8.1×10^7	120
CS-Rif 4	1.8×10^9	70	1.4×10^9	72	1.2×10^9	80	8.4×10^8	85
CS-Rif 6	2.3×10^9	75	1.8×10^9	75	1.6×10^9	90	7.6×10^8	105
CS-Rif 3	1.8×10^9	70	1.4×10^9	70	1.3×10^9	80	6.4×10^8	85
CS-Rif 7	1.9×10^9	75	1.4×10^9	75	1.2×10^9	90	6.2×10^8	105
CS-Rif 9	1.7×10^9	70	1.3×10^9	70	1.2×10^9	80	7.4×10^8	85

Figure.1 Growth rate of CS mutants in LAPTg at 37°C

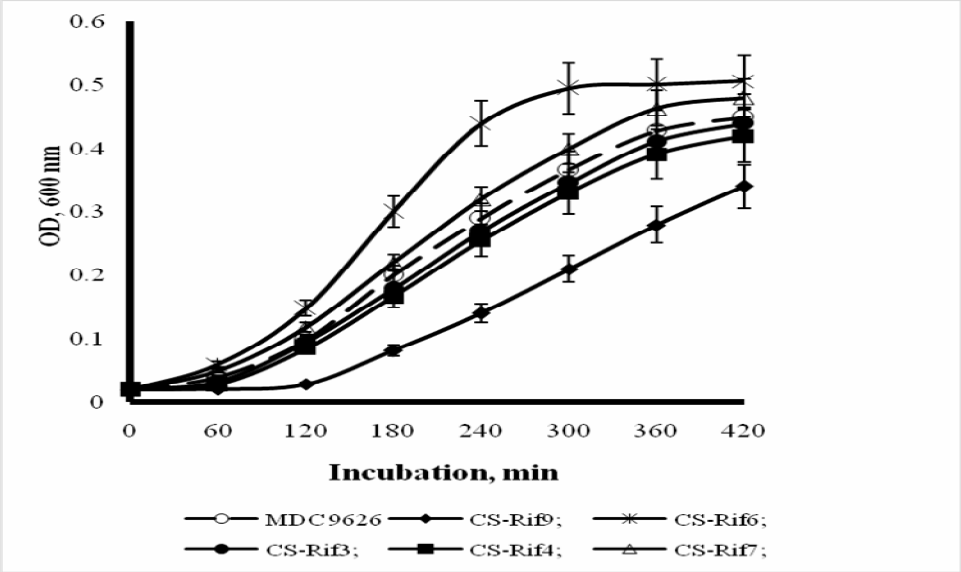
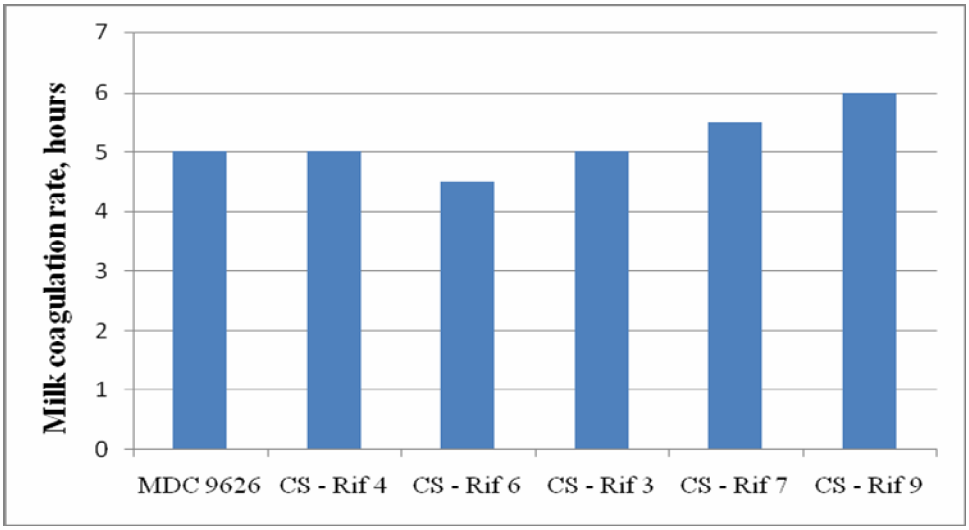


Figure.2 The rate of milk`s coagulation by CS mutant starters at 37°C



Thus, the *rif* mutations can be used not only for expanding shelf life of dairy products, but also for obtaining strains with improved technological characteristics of starter cultures and organoleptic properties of ready - to- use products.

In conclusion, among rifampicin-resistant mutants of LAB by frequency of about, 1.0 % is possible to isolate CS mutants simultaneously possessing of high specific growth rate, rate of milk fermentation. Conditionally lethal CS *rif* mutations can be used for expanding of the shelf life of dairy products at fridge temperatures as well as for improving technological characteristics of starter cultures.

Reference

Beal, C., Skokanova, J., Latrille, E., Martin, N., Corrieu, G. 1999. Combined effects

of culture conditions and storage time on acidification and viscosity of stirred yoghurt. *J. Dairy Sci.*, 82: 673–781.

Elisabetta, C., Clelia, P., Salvatore, M.T., Francesco, F., Adelfia, T., Giorgio, C., Silvio, B., Gianluca, De B., Pietro, A. 2009. Phenotypes and gene expression profiles of *Saccharopolyspora erythraea* rifampicin-resistant (rif) mutants affected in erythromycin production. *Microbial Cell Factories*, 8(18): 1–15.

Garibyan, L., Huang, T., Kim, M., *et al.* 2003. Use of the *rpo*B gene to determine the specificity of base substitution mutations on the *Escherichia coli* chromosome. *DNA Repair* (Amst), 2: 593–608.

Hovhannisyan, H.G., Barseghyan, A.A., 2015. The influence of rifampicin resistant mutations on the biosynthesis of exopolysaccharides by strain *Escherichia coli K-12 lon. Appl. Biochem. Microbiol.*, 51(5): 546–550.

Hovhannisyan, H.G., Barseghyan, A.A., Grigoryan, N.G., Topchyan, A.V. 2010. Genetic improvement of technological characteristics of starters for fermented milk products. *Appl. Biochem. Microbiol.*, 46(4): 395–399.

Hua,X.T., Wang, C., Huang, L., *et al.* 2010. Mutation rate and spectrum of spontaneous mutations of *Deinococcus radiodurans* under rifampin stress. *J. Nucl. Agric. Sci.*, 24: 1166–1171.

Huys, G., D'Haene, K., Swing, J. 2002. Influence of the culture medium on antibiotic susceptibility testing of food-associated lactic acid bacteria with the agar overlay disc diffusion method. *Lett. Appl. Microbiol.*, 34**:** 402–406.

Ingham, C.J., Furneaux, P.A. 2000. Mutations in the *β*subunit of the *Bacillus subtilis* RNA polymerase that confer both rifampicin resistance and hypersensitivity to NusG. *Microbiology*, 146: 3041–3049.

Jin, D.J., Gross, C.A. 1989. Characterization of the pleiotropic phenotypes of rifampin-resistant *rpoB* mutants of *Escherichia coli. J. Bacteriol.*, 171(9): 5229–5231.

Kogoma, T. 1994. *Escherichia coli* RNA polymerase mutants that enhance or diminish the SOS response constitutively in the absence of RNase HI activity. *J. Bacteriol.*, 176(5): 1521–1523.

Lai, C., Xu, J., Tozawa, Y., *et al.* 2002. Genetic and physiological characterization of rpoB mutations that activate antibiotic production in *Streptomyces lividans*. *Microbiology*, 148: 3365–3373.

Lengeler, J.W., Drews, G., Schlegel, H.G., 1999. Biology of the prokaryotes. Georg Thieme, Verlag, 955.

Maughan, H., Galeano, B., Nicholson, W.L. 2004. Novel *rpo*B mutations conferring rifampin resistance on *Bacillus subtilis*: global effects on growth, competence, sporulation, and germination. *J. Bacteriol.*, 186: 2481–2486.

Shafiee, G., Mortazavian, A.M., Mohammadifar, M.A., Koushki, M.R., Mohammadi, A., Mohammadi, R. 2010. Combined effects of dry matter content, incubation temperature and final pH of fermentation on biochemical and microbiological characteristics of probiotic fermented milk. *Afr. J. Microbiol. Res.*, 4(12): 1265–1274.

Tamime, A.Y., Robinson, R.K. 2011. Yoghurt: science and technology. Woodhead Publishing Ltd., England. 345 Pp.

Telenti, A., Imboden, P., Marchesi, F., Lowrie, D., Cole, S., Colston,M.J., Matter, L., Schopfer, K., Bodmer, T. 1993. Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis*. *Lancet*, 341: 647–650.