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Acidophilus Milk Shelf-life Prolongation by the Use of Cold Sensitive Mutants of *Lactobacillus acidophilus* MDC 9626

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Abstract

Background and Objective: The shelf-life of acidophilus milk fermented by probiotic culture *Lactobacillus acidophilus* is limited due to acidification caused by continued organic acid formation at low temperatures. Increasing of titrable acidity in turn causes reducing of the total viable count of probiotic bacteria. To overcome acidification we suggested to use cold-sensitive mutants of *Lactobacillus acidophilus*, with limited metabolism at low temperatures. In order to facilitate the selection of cold sensitive mutants, it was decided to use Rifampicin and Streotomycin mutations affecting thermostability of the key molecules of cell metabolism the RNA polymerase and ribosome, respectively.

Material and Methods: Ultra violet mutagenesis was used to enhance the yield and diversity of rifampicin and streptomycin resistant mutants of *Lactobacillus acidophilus*. To perform negative selection of cold sensitive mutants, antibiotic resistant colonies replica plated and incubated at 23ºC. The growth rate, milk fermenting rate, titratable acidity were measured.

Results and Conclusion: Among tested resistant to either rifampicin or streptomycin clones with frequency mean of 1.0%, ten mutants were isolated which have lost the ability to grow at minimal temperature. Fermented with cold-sensitive mutants of *Lactobacillus acidophilus* milks, during storage in the refrigerator, almost twice as long retained high amount of probiotic bacteria and low titratable acidity as compared to the parent strain. Thus, direct relationship between temperature sensitivity of the starter and shelf life of acidophilic milk was confirmed. Rifampicin and Streptomycin resistant mutations are powerful tools for selection of cold-sensitive dairy starters for preparing dairy fermented products with long shelf-life.

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1. Introduction

Acidophilus milk is one of well-known dairy probiotic products produced in many countries over a century as a functional food. Acidophilus milk (or fermented milk) contains live cultures of *Lactobacillus (L.) acidophilus* that definitely has tremendous health benefits due to its high probiotic properties. The shelf life of all type of fermented milk products are limited due to continuation of metabolic activity during storage in the cold, the titrated acidity increases, which in turn leads to a drop in the number of living cells [1-3].

The global food industry is constantly exploring new approaches to improve product quality to fulfil consumers' demand for good taste and texture for a long shelf-life, therefore there is a need for constant search and development of new starter cultures with novel properties. Wild type strains may have unique industrial properties, but to fully employ their potential, specific actions are often required. In other cases, it can be of interest to improve strains which already have established industrial applicability or to reduce/eliminate an unwanted property [4-8]. The adaptation often used to make microorganisms more tolerant to environmental stresses has temporary effect. On the other hand, the genetically determined changes have permanent features [9-11].

Modern high recombinant DNA technology would be an ideal way to improve Lactic acid bacteria (LAB) properties by restrictive food legislation and consumers concerns with genetically modified food ingredients [12]. Thus, the main efforts to improve LAB strains for industrial application are currently based on classical methods for strain improvement such as random mutagenesis, directed evolution and dominant selection [13,14].

Random mutagenesis extensively used in the food industry is based on the introduction of random mutations into the genome of interest, characterization of a large library of variants, and selection of strains with the desired property for further use [15,16].

Mutations concerning processes of transcription and translation may be promising for genetic improvement of industrial characteristics of lactobacilli. It is known that mutations causing resistance to rifampicin (*rif*) and streptomycin (*str*) concern β subunit of RNA polymerase and S12 protein from small subunit of ribosome, respectively [17,18]. A characteristic feature of *rif* and *str* mutations is high pleiotropic of phenotypic expression. They are able to cause variant reading genetic information in processes of transcription and/or translation practically of every gene that results in simultaneous alterations of a whole spectrum of features of bacterial cells: colony morphology, growth rate, sensitivity to temperature, requirements in growth factors, etc [19].

The microorganisms growth (especially in rich media) at extreme temperatures limited by cold or heat sensitivity of one or more key protein(s) are involved in global cellular processes such as DNA replication, transcription, translation or cell division [20]. The main cause of deterioration of fermented milk products can be the metabolic activity of starter cultures at low temperatures [21-23]. The use of cold sensitive mutant starters with metabolic activity restriction at low temperatures could be an alternative solution for expanding shelf life of fermented dairy products [13,14].

The aim of this work is to demonstrate mutational restriction impact of *L. acidophilus* growth at low temperatures by affecting the termostability of RNA polymerase and ribosome, the key components of protein synthesizing apparatus, could reduce acidification rate and thus expand the shelf life of acidophilus milk.

2. Materials and Methods

2.1. The bacterial cultures

The *L. acidophilus* MDC 9626 was obtained from the Armenian National Microbial Depository Center [24].

2.2. Media

Skimmed milk; LAPTg was made up of yeast extract, 10 g, peptone, 15 g, tryptone, 10 g, glucose 10 g, Tween, 1ml per 1 L of distillated water, for solid medium, 1.5 % Bacto-agar was included, Tryptose agar (T-agar) [Merck, Germany], Rifampicin and Streptomycin purchased from "Serva" (Germany). Sodium phosphate buffer (pH 6.8).

2.3. Mutagenesis and selection of antibiotic resistant mutants

LABs were grown at 37°C in LAPTg to optical density up to OD 0.6; cells were washed twice and harvested twice by centrifugation at 5000×g for 15 min and transferred in phosphate buffer. Aliquots of cell suspensions (2 ml) were transferred to sterile petri dishes and irradiated with UV-light (254 nm) for 20 seconds [25]. Irradiated cells were diluted ten fold in fresh LAPTg broth and grown at 37°C for 4 h to permit 3- 4 division cycles. Cells were plated on LAPTg agar containing 100 µg ml^{-1} of appropriate antibiotic and incubated at 37°C for obtaining antibiotic resistant colonies.

2.4. Cold sensitive mutants screening

Cold sensitive (CS) mutants were screened by replica plating of *str* and *rif* colonies and cultivating at 23°C. The colonies,that did not grown on the replica plates wereconsidered to be cold sensitive and picked up from the master plate for further study [26].

2.5. Acidophilus milk preparation

Pasteurized milk was inoculated by 0.01% (w v^{-1}) *L. acidophilus* CS starters and aliquots 50 ml aseptically distributed in 100 ml bottles (one bottle for each sampling time), then fermentation followed until the milk coa-gulation. Fermented Acidophilus milks were kept at 5°C for 28 days for the performance of microbiological and chemical analysis at 7 day interval. At each sampling day, one bottle was withdrawn and after vigorous shaking, 1ml of its content placed into 9 ml of physiological solution, appropriate dilutions were made and subsequently plated onto LAPTg agar and incubated aerobically at 37°C. The grown bacterial colonies were counted and multiplied by dilution factor and the results expressed as CFU ml^{-1}. The remainder milk was used for determination of titratable acidity.

2.6. Milk coagulation rate determination

Pasteurized skimmed milk samples were inoculated by cold sensitive starters and incubated at 37°C, then tested for coagulation every 30 min.

2.7. Growth rate

Overnight cultures diluted 20 times in fresh LAPTg broth and growth at 37°C in 250 ml Erlenmeyer flasks, with shaking at 200 rpm. The turbidity was measured at OD_{600} every 30 min.

2.8. Milk coagulation rate

Samples of sterile skim milk by volume 1.8 ml were inoculated by 0.2 ml of overnight broth cultures and incubated at 37°C, and checked for clotting every 30 min.

2.9. Titratable acidity assay

The titratable acidity of the fermented milk was performed according to Thorner [23]. Titratable acidity (^oT) expressed as a percentage of lactic acid which was neutralized with 0.1 N NaOH, until a pink color appeared in the presence of phenolphthalein.

2.10. Optical density (OD) assay

Bacterial suspension concentration was determined by measuring OD_{600} in spectrophotometer.

2.11. Statistical analysis

Statistical analysis was performed using SPSS software for windows (Version 16) (SPSS Inc. Chicago, IL and USA). Mean and Standard deviation was used to describe data. Fisher's range test was used to determine differences between tested groups. $P \leq 0.05$ was considered as significant. All experiments were replicated three times.

3. Results and Discussion

3.1. Selection of *L. acidophilus* CS starters by use of *rif* and *str* mutations

After UV mutagenesis of *L. acidophilus* 486 colonies formed on LAPTg agar with 100 μg ml^{-1} of rifampicin and 446 colonies with 100 μg ml^{-1} of streptomycin were replica plated on agar with the appropriate antibiotics and incubated at 23°C. The colonies which are not growing on replica plates were suggested as CS and picked from master plates for further investigation. The frequency of obtaining CS variants among Rif mutants was about 1.0% and among Str about 1.1%. Five of Rif and five of Str mutants, which didn't grow at minimal temperatures for serial passages, were isolated for further investigation.

The growth temperature range of *L. acidophilus* MDC 9626 CS mutants in LAPTg broth was investigated (Table 1).

As seen in table 1, due to *rif* and *str* mutations the minimal temperatures of growth shift-up 4 to 9 degrees, but the optimal growth temperature range of all CS mutants remained unchanged.

Table 1. Determination of the temperature ranges of CS mutants growth in LAPTg

Strains	Growth temteratures, °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
CS-Str1	29	37 - 42	45
CS- Str 2	24	37 - 42	48
CS- Str 3	28	37 - 42	48
CS- Str 4	24	37 - 42	48
CS- Str 5	29	37 - 42	48

3.2. The growth rate of *L. acidophilus* CS mutants in LAPTg broth

The growth curves of CS cultures in LAPTg broth at 37°C are presented in Figure 1. From growth curves presented in Figure 1, it can be seen that *rif* and *str* mutations also affect the growth rate of *L. acidophilus* MDC 9626. Thus, the mutants CS-Rif6 and CS-Str2 grow significantly faster than parental strains when the CS-Rif3 and CS-Str1 growth slows down remarkably. The other growth rates were not significantly different from their parental strains. The mutants CS-Rif3 and CS-Str5 accumulated 2-3 times more biomass than parental strains which is very important for the starter industry.

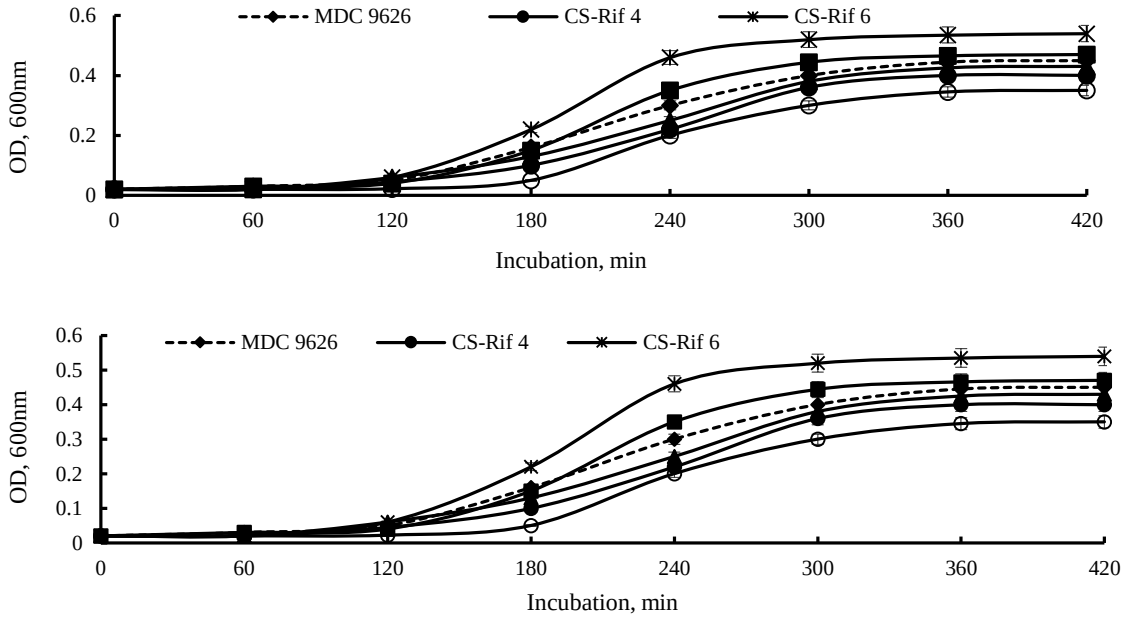


Figure 1. Kinetics of growth of *L. acidophilus* CS-cultures in LAPTg at 37°C

3.3. The rate of milk coagulation by CS mutants

The rate of milk coagulation by CS starters was defined at 37°C. The comparative time of milk coagulation rates are presented on (Figure 2).

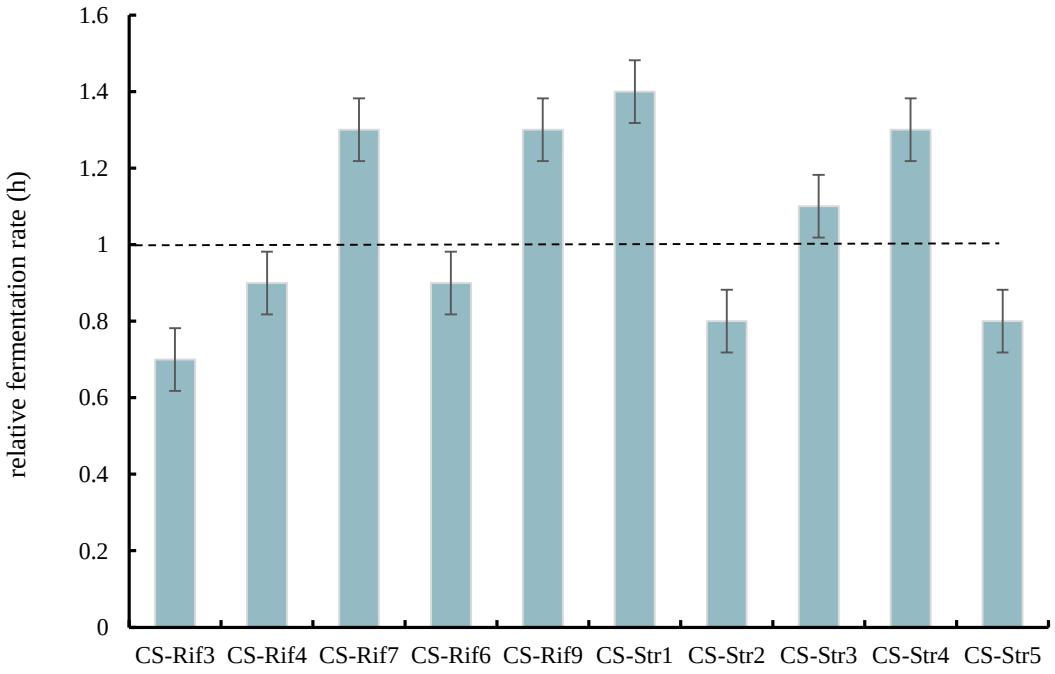
As it can be seen in Figure 2, some *rif* and *str* mutations, along with cold sensitivity, also have a significant effect on the rate of coagulation of milk. Of particular interest are the mutants CS-Rif7 and CS- Str1 whose coagulation rate increased 1.3 and 1.4 times, respectively. In the remaining mutants, the coagulation rate either decreased or was slightly different from the parent strain.

3.4. Investigation of milk fermentation temperature profile of CS mutants

Milk fermentation by CS starters was studied at temperature ranges of 20-48°C (Table 2).

As seen in table 2, the strains resistant to rifampicin CS-Rif 4, CS-Rif 3, CS-Rif 9 and resistant to streptomycin CS-Str1, CS-Str3 and CS-Str5 lost the ability to ferment milk below 30°C. The remainder mutant were able to coagulate milk at 24°C. Only one mutant, the CS- Rif9 could not ferment milk at 48°C.

Thus, the *rif* and *str* mutations shift-up the minimal temperature of milk fermentation of *L. acidophilus* MDC 9626 by 4-9 degrees.



The dashed line represents the milk coagulation rate by wild strain taken as unit.

Figure 2. The relative milk coagulation rates by *L. acidophilus* CS starters at 37°C.

Table 2. Milk coagulation temperature ranges of CS starters

	Temperature, °C							
Strains	20	24	27	30	37	42	45	48
MDC 9626	+	+	+	+	+	+	+	+
CS-Rif 4	-	-	-	+	+	+	+	+
CS-Rif 6	-	+	+	+	+	+	+	+
CS-Rif 3	-	-	-	+	+	+	+	+
CS-Rif 7	-	+	+	+	+	+	+	-
CS-Rif 9	-	-	-	+	+	+	+	+
CS- Str 1	-	-	-	+	+	+	+	-
CS- Str 2	-	+	+	+	+	+	+	+
CS- Str 3	-	-	-	+	+	+	+	+
CS- Str 4	-	+	+	+	+	+	+	+
CS- Str 5	-	-	-	+	+	+	+	+

+ Coagulated, - Not coagulated

3.5. Shelf life determination of dairy products

The live bacterial count and titratable acidity are the main criteria for definition of fermented dairy probiotics quality [21,27]. Fermented by CS starters acidophilus milks were stored at 5°C and live microbial count and titratable acidity were measured every week for 28 days (Table 3). On the next day following storage, the titratable acidy in the sample fermented with *L. acidophilus* was higher (41ºT) than others due to its ability to retains metabolic activity upon cooling to 20ºC, whereas in CS mutants it ceases earlier at higher temperatures. On the 7th day of storage, the changes in the amount of bacteria and titratable acidity in the product fermented by *L. acidophilus* already are visible. The mutants, which have higher growth $T_{min,}$ have kept higher amounts of living bacteria and have had lower acidification rate for 21 days in cold storage.

At the end of storage, the viable count of CS-Rif3 and CS-Str5 mutants dropped only 0.6 log and titratable acidity reaches to 41ºT and 49ºT, respectively, whereas the titer of wild bacteria dropped up to 4.2 log and titratable acidity reached to 72ºT.

As expected, the probability of selecting cold-sensitive starters among the *Rif* and *Str* mutants were very high. Thus, the UV induced mean yields of *Rif* and *Str* mutants were 1.8×10^{-5} and 2.9×10^{-6}, respectively, whereas among the both type mutants the frequency of obtaining CS variants was approximately equal to 1.1×10^{-2}. In these cells, *rif* and *str* mutations alter the structure of cell key components; β subunit of RNA polymerase and S12 protein of ribosome and turn them nonfunctional in minimal temperature. Beside shift-up of growth temperature, several *rif* and *str* mutations due to their pleiotropy, cause other phenotypical changes such as specific growth rate, milk fermentation rates, titratable acidity and cells viability at low temperatures. It has been shown that *rif* and *str* mutations also possess high pleiotropy by interfering in a variety of physiological processes of, *Escherichia coli*: rate of growth, the ability of mutants to support the growth of various bacteriophages; the ability to maintain the F' episome; interaction with mutant alleles of other genes and technological properties of LAB [19,28-32]. The use of *rif* and *str* mutations significantly triggered the effectiveness of cold sensitive mutants selection.

Experiments confirmed our prediction that dairy starter's metabolic activity at storage temperatures are the main reason for short shelf-life of fermented dairy products. Acidophilus milk fermented by the use of cold sensitive starters, restricted metabolism at low temperatures and has had longer shelf life and contain higher amounts of living probiotics.

The rif and str mutations can be used not only for expanding shelf life of acidophilus milk, but also for improved technological characteristics of lactic acid bacteria. The high rate of growth and high biomass accumulation are very important in dairy starters and probiotics manufacturing.

Table 3. The nature of changes in the number of living cells and titratable acidity in acidophilic milks prepared using CS starters at 5ºC

	Days of Storage									
	1		7		14		21		28	
Strains	CFU ml^{-1}	˚T	CFU ml^{-1}	˚T	CFU ml^{-1}	˚T	CFU ml^{-1}	˚T	CFU ml^{-1}	˚T
L.acidophilus	1.7×10^{9}	41	1.2×10^{9}	49	2.4×10^{8}	56	11×10^{7}	68	8.1×10^{6}	72
CS-Rif 4	1.8×10^{9}	38	1.4×10^{9}	42	7.2×10^{8}	44	2.4×10^{8}	49	8.4×10^{7}	61
CS-Rif 6	2.3×10^{9}	36	1.8×10^{9}	41	1.6×10^{8}	44	7.6×10^{8}	48	7.6×10^{7}	62
CS-Rif 3	3.8×10^{9}	30	3.7×10^{9}	31	1.3×10^{9}	31	3.5×10^{9}	32	6.4×10^{8}	41
CS-Rif 7	1.9×10^{9}	36	1.4×10^{9}	42	1.2×10^{8}	45	6.2×10^{8}	51	6.2×10^{7}	63
CS-Rif 9	1.7×10^{9}	34	1.3×10^{9}	36	7.2×10^{8}	40	1.4×10^{8}	48	7.4×10^{7}	62
CS- Str 1	1.3×10^{9}	36	1.2×10^{9}	38	6.1×10^{8}	39	2.4×10^{8}	51	8.4×10^{7}	59
CS- Str 2	2.3×10^{9}	34	1.9×10^{9}	38	7.7×10^{8}	41	1.7×10^{8}	53	7.7×10^{7}	62
CS- Str 3	1.8×10^{9}	36	1.4×10^{9}	39	1.3×10^{8}	42	6.4×10^{8}	58	9.4×10^{7}	67
CS- Str 4	2.0×10^{9}	31	1.3×10^{9}	38	8.2×10^{8}	41	2.4×10^{8}	52	7.4×10^{7}	64
CS- Str 5	3.5×10^{9}	31	3.5×10^{9}	32	1.2×10^{9}	35	3.4×10^{9}	36	6.1×10^{8}	49

4. Conclusion

There is a relationship between cold-sensitivity and maintenance of bacterial viability and acidity of fermented probiotic acidophilus milk during cooling and cold storage. Shelf life of acidophilus milk fermented by CS mutants determined by viable count of probiotic bacteria and titratable acidity is significantly larger than the *L. acidophilus* parental strains. In order to extend the shelf-life of fermented dairy products, *rif* and *str* mutations can be used to improve the efficiency of selection of cold-sensitive starters. These mutations can also be used for intensification of milk fermentation process at optimal conditions.

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6. Conflict of Interest

The authors declare no conflict of interest

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افزایش طول انباری شیر اسیدی با استفاده از لاکتوباسیلوس اسیدوفیلوس MDC 9626 جهش یافته حساس به سرما

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چکیده

سابقه و هدف: عمر انباری شیر اسیدی تخمیر شده توسط پروبیوتیک لاکتوباسیلوس اسیدوفیلوس به‌دلیل اسیدی شدن ناشی از تداوم تشکیل اسید آلی در دمای پایین، محدود است. افزایش اسیدیته قابل تیتراسیون به‌نوبه خود موجب کاهش تعداد کل باکتری‌های پروبیوتیک زنده می‌شود. برای غلبه بر اسیدی شدن، پیشنهاد ما استفاده از لاکتوباسیلوس اسیدوفیلوس جهش یافته حساس به سرما و با متابولیسم محدود شده در دمای پایین می‌باشد. به‌منظور تسهیل در انتخاب جهش‌های حساس به سرما، تصمیم گرفته شد از ریفامپیسین و استرپتومایسین جهش یافته استفاده شود که به ترتیب بر پایداری حرارتی مولکول‌های کلیدی متابولیسم سلولی به ترتیب، RNA پلیمراز و ریبوزوم تاثیر دارند.

مواد و روش‌ها: برای افزایش عملکرد و تنوع باکتری لاکتوباسیلوس اسیدوفیلوس جهش یافته مقاوم به ریفامپیسین و استرپتومایسین از جهش زایی با پرتو فرا بنفش استفاده شد. برای انجام انتخاب منفی جهش‌های حساس به سرما، کلنی‌های مقاوم به آنتی بیوتیک با روش رپلیکا پلیتینگ مشخص شده و در دمای ۲۳ درجه سانتیگراد گرمخانه گذاری شدند. سرعت رشد، سرعت تخمیر شیر، اسیدیته قابل تیتراسیون اندازه گیری شد.

یافته‌ها و نتیجه‌گیری: در میان کلنی‌های مورد آزمون انواع مقاوم به ریفامپسین و استرپتومایسین با میانگین بسامد ۱٪، ده جهش یافته جدا شدند که توانایی رشد در پایین ترین دما را از دست داده بودند. در مدت نگهداری در یخچال، شیر اسیدی تخمیر شده با جهش یافته‌های حساس به سرما، در مقایسه با سویه اولیه، (والد)، تقریبا به میزان دو برابر تعداد باکتری پروبیوتیک بیشتر و اسیدیته قابل تیتراسیون کمتری را موجب شدند. بنابراین رابطه مستقیم بین حساسیت دمایی آغازگر و عمر انباری شیر اسیدی تأیید شد. جهش یافته‌های مقاوم به ریفامپیسین و استرپتومایسین ابزار قدرتمند برای انتخاب آغازگرهای حساس به سرما برای تهیه محصولات تخمیر شده لبنی با عمر انباری طولانی می باشند.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ تعارض منافعی وجود ندارد.

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واژگان کلیدی

- شیر اسیدوفیلوس
- جهش یافته حساس به سرما
- جهش یافته مقاوم به ریفامپیسین و استرپتومایسین
- عمر انباری
- *لاکتوباسیلوس اسیدوفیلوس*

***نویسنده مسئول**

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