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 titratable 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 Streptomycin 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 23°C. 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.

Conflict of interest: The authors declare no conflict of interest.

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 acidification caused by continued 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 distilled 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 5000xg 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 grow on the replica plates were considered 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 coagulation. 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 OD₆₀₀ 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 Thorne [23]. Titratable acidity (°T) 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≤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

<table>
<thead>
<tr>
<th>Strains</th>
<th>$T_{\text{min}}$</th>
<th>$T_{\text{opt}}$</th>
<th>$T_{\text{max}}$</th>
</tr>
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<tbody>
<tr>
<td>MDC 9626</td>
<td>20</td>
<td>37 - 42</td>
<td>48</td>
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<tr>
<td>CS-Rif 4</td>
<td>28</td>
<td>37 - 42</td>
<td>48</td>
</tr>
<tr>
<td>CS-Rif 6</td>
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<td>37 - 42</td>
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<tr>
<td>CS-Rif 3</td>
<td>29</td>
<td>37 - 42</td>
<td>48</td>
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<td>45</td>
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<tr>
<td>CS-Rif 9</td>
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<td>37 - 42</td>
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<tr>
<td>CS-Str1</td>
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<tr>
<td>CS-Str 5</td>
<td>29</td>
<td>37 - 42</td>
<td>48</td>
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</tbody>
</table>

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](image-url)
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>
<thead>
<tr>
<th>Strains</th>
<th>Temperature, °C</th>
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<tbody>
<tr>
<td></td>
<td>20</td>
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<tr>
<td>MDC 9626</td>
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<tr>
<td>CS-Rif 4</td>
<td>-</td>
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<tr>
<td>CS-Rif 6</td>
<td>-</td>
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<tr>
<td>CS-Rif 3</td>
<td>-</td>
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<td>CS-Rif 7</td>
<td>-</td>
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<td>CS-Rif 9</td>
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<tr>
<td>CS-Str 1</td>
<td>-</td>
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<td>CS-Str 2</td>
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<td>CS-Str 3</td>
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<tr>
<td>CS-Str 4</td>
<td>-</td>
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<tr>
<td>CS-Str 5</td>
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</table>

+ 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 acidity in the sample fermented with *L. acidophilus* was higher (41°C) than others due to its ability to retain metabolic activity upon cooling to 20°C, 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<sub>min</sub>, 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 reached to 72°C. 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<sup>e</sup> 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>
<thead>
<tr>
<th>Table 3. The nature of changes in the number of living cells and titratable acidity in acidophilic milks prepared using CS starters at 5°C</th>
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<tr>
<td><strong>Strains</strong></td>
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<td><em>L. acidophilus</em></td>
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<td>CS-Rif 4</td>
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<td>CS-Rif 6</td>
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<td>CS-Rif 9</td>
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<td>CS- Str 1</td>
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<td>CS- Str 2</td>
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<td>CS- Str 4</td>
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<td>CS- Str 5</td>
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</table>
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.

5. Acknowledgements

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6. Conflict of Interest

The authors declare no conflict of interest

References


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

عطرضا گودرزی، هرامیا هوودیاسان، گوهر گربکوران و اندرانیک بارسیان

نگارنده سلولی سرما و اندرانیک بارسیان

چکیده

محصولات سلامتی: علمی ایران

چکیده

اطلاعات مفاد

دریافت یک ماه ۱۳۹۶

دریافت اولیه ۱۳۹۶

دریافت مرحله‌ای ۱۳۹۶

تاریخ پذیرش ۱۳۹۶

پذیرش اولیه ۱۳۹۶

چکیده

مانکن‌های آزمایشی گاو‌های بزرگسال به مدت یک ماه در انباری شده بودند که در انتهای آن‌ها به دستگاه‌های آزمایشی در دو گروه (ارزی و آزمایشی) تقسیم شدند. در گروه ارزی، شیر اسیدی و یک نگهداری مالینه با چسبانده‌های رپلیکا به همراه افزایش حساس به سرما در دو گروه ارزی و آزمایشی به صورت ساده انجام شد. نتایج نشان داد که تیتراسیون به میزان ۲/۲ در گروه‌های ساده داشت. نتایج نشان داد که تیتراسیون به میزان ۲/۲ در گروه‌های ساده داشت.