The Use of Str Mutations for Enhancement of Hydrogen Peroxide Formation by *Lactobacillus Delbrueckii* MH-10 at Refrigeration

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**Abstract:** The strains of *Lactobacillus delbrueckii* subsp. lactis widely used in food preservation due to ability produce high amount of hydrogen peroxide at refrigerator temperatures to inhibit food-borne pathogens and psychrophilic spoilage microorganisms. In order to improve of bio-preservation efficacy of *L. delbrueckii* MH 10 mutations causing resistance to streptomycin (str) were used. Among UV-mutagenized population of *L. delbrueckii* three *str* mutants producing high amounts of H$_2$O$_2$ were selected. *Str* mutants produced significant amounts of hydrogen peroxide 50-60 μg/ml in sodium phosphate buffer (0.2 M, pH 6.5) and in beef broth (BB) at 5 °C for 5 days submerged cultivation without of growth. Evaluation mutants antibacterial activity at refrigeration temperatures against food-borne pathogen *Escherichia coli* O157:H7 revealed elimination of pathogen total number up to practically undetectable amount for 3 days. In case of solid-state cultivation on agar-based medium, disks soaked by mutant cells suspensions formed larger inhibitory zones on *E. coli* O157:H7 lawn for one-day cold exposition. The size of inhibition zone depends on concentration of LAB cells. *Str* mutants *L. delbrueckii* reduced initial amount 2 × 10$^5$ of *E. coli* O157:H7 in ground beef up to 3 log for 3 days of solid-state cocultivation when the wild strain reduced only 2 log. The application of *L. delbrueckii* mutants did not cause any changes in sensory characteristics of ground beef, moreover promotes expanding of shelf-life due to inhibition of psychrophilic spoilage microorganisms.

**Key words:** Biopreservation, *Lactobacillus delbrueckii*, Str mutations, refrigerated temperatures, hydrogen peroxide, *E. coli* O157:H7.

**1. Introduction**

For ground meat shelf life prolongation, synthetic chemicals have been traditionally used to inhibit resident pathogenic and spoilage microorganisms in refrigerated products. The increasing consumer concerns of potential health risks associated with some of synthetic preservatives has led researchers to evaluate the opportunity of using natural bio-preservatives such as Lactic Acid Bacteria (LAB) selected for their inhibitory activity towards undesirable microorganism [1, 2]. Lactic acid bacteria produce a wide range of inhibitory compounds such as organic acids, hydrogen peroxide, diacetyl and bacteriocins and thus, expanding shelf life and increasing food safety and have Generally Recognized As Safe (GRAS) status [3-7]. The inhibitory actions of LAB toward food-borne pathogens and spoilage organisms in non-processed foods occur during entire storage period by continuous production of inhibitory compounds instead of a one-time reduction, as occurs with antimicrobial substances interventions. It has been shown that for bio-preservation the most effective are LABs able to produce hydrogen peroxide at refrigerated temperatures in absence of growth [8-10].

Most raw foods are contaminated with pathogenic and spoilage microorganisms. Ground beef products are common sources of *E. coli* O157:H7 and its reduction is an important concern in the beef industry. It was shown LAB impact on *E. coli* O157:H7 viability in ground beef and the sensory properties of
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These products [11-13]. The species L. delbrueckii subsp. lactis more of all used in food preservation, but hydrogen peroxide production is variable amongst strains [14, 15]. But the use of limited number of LAB strains may cause decreasing of treatment efficacy due to accumulation of deleterious mutations and/or adapting of pathogens to antibacterial substances which are produced [3]. In order to enhance biopreservation efficacy LAB mutant producing high amount of antimicrobial substances should be selected and methods developed for their cultivation and application to food [16-18]. Because hydrogen peroxide production plays the major role in elimination of the pathogens at refrigerator storage, its levels should be assessed for newly selected strains. There are not methods for direct selection of antimicrobials producing mutants, so we need in methods to enhance the likelihood of this mutants occurrence by help of mutations affecting global regulatory network of cell. Enhancement of secondary metabolites yield is problematic issue in biotechnology. Earlier we successfully used ribosome and RNA polymerase mutations for improvement of technological characteristics and enhancement secondary metabolites (polysaccharides, aroma substances, etc.) yields of industrial microorganisms [19, 20].

The objectives of this research were selection of str mutants L. delbrueckii MH 10 possessing high hydrogen peroxide production ability at cold storage and evaluation their impact on E. coli O157:H7 in ground beef during refrigerated storage.

2. Materials and Methods

2.1 The Bacterial Cultures

The Lactobacillus delbrueckii MH 10 from human origin was isolated and identified in our laboratory by Hovhannisyan and Pashayan (2010) and deposited in Armenian National Microbial Depository Center (MDC) under code MDC 9626. Food born pathogen Escherichia coli O157:H7 MDC 5003 used in this study was from the MDC.

2.2 Media

LAPTg medium [21] (yeast extract—10 g, peptone—15 g, tryptone—10 g, glucose—10g, Tween—1 mL) and modified LAPTg without of yeast extract and lower concentration (5 g) of glucose. For solid medium, 1.5% Bacto-agar was included. Nutrient broth (NB) [Serva, Germany], Tryptose agar (T-agar) [Merck, Germany]. Beef broth (BB) was prepared by dissolving of 20 g Sigma beef extract powder in 1 L distilled water. Fresh ground meat was purchased from butchers in Armenia and transported to the laboratory using a refrigerated box. Sodium phosphate buffer (pH 6.5). Physiological saline—0.9% NaCl. Merckoquant Peroxide Test strips [Merck, Germany].

2.3 Mutagenesis and Selection of Antibiotic Resistant Mutants

L. delbrueckii MH 10 was grown at 37 °C in LAPTg to optical density up to OD 0.6 cells were harvested by centrifugation at 12,000 × g for 15 min and transferred in PBS. Aliquots of cell suspensions (2 mL) were poured into sterile petri dishes and irradiated with UV—light (254 nm) for 5, 10, 20 and 40 sec. Irradiated cells diluted tenfold into fresh LAPTg broth and were grown at 37 °C for 4 h permit to undergo 3-4 division cycles. For obtaining streptomycin resistant mutants cells were plated on LAPTg agar containing 100 μg/ml of the antibiotic and incubated at 37 °C till colonies appearance.

2.4 Bacteriological Analysis

Bacterial count in liquid media was made using standard methods. For enumeration of E. coli and lactobacilli in ground beef 1 g infected meat sample was inoculated in 9 mL of sterile physiological, homogenized, made serial ten-fold dilutions and plated on Tryptose and MRS agars for determination of E. coli and LAB counts, respectively.
2.5 Hydrogen Peroxide Assay

Hydrogen peroxide concentration measured by Merckoquant Peroxide Test strips with measuring ranges 0.5-2-5-10-25 and 1-3-10-30-100, according to the manufacturer instruction.

2.6 Potentiometric and Titratable Acidity

The pH was measured at room temperature, using a digital pH meter. Titratable acidity expressed as a percentage of lactic acid was measured by titrating 9 mL of the sample (where added 3 spots of phenolphthalein) with 0.1 N NaOH, until a pink color appeared.

2.7 Agar Disk Diffusion Method

Agar disk diffusion method was used to evaluate the antimicrobial effect of LAB suspensions. E. coli O157: H7 culture grown in NB broth for 18 h at 37 °C diluted to concentration of 10^7 cells/mL and spread onto Tryptose agar. The paper discs (diameter, 5 mm) were soaked with LAB culture liquids and placed on the test culture lawn. After 2 h exposition in cold the plates were incubated at 37 °C for 18 h and examined for size of clear inhibitory zones.

2.8 Quantification of Antimicrobial Activity of LAB In cold Cultivation

Lactobacilli were grown in LAPTg broth for 18 h at 37 °C divided in four 10 mL aliquots, centrifuged at 12,000 × g for 10 min and each pellet resuspended in 10 mL of cold medium; sodium phosphate buffer and beef broth and incubated at 5 °C. Every two days for 7 days and then each week antimicrobial activity, hydrogen peroxide amount, OD600 and pH of the cell cultures were determined.

2.9 Submerged Cocultivation of LAB along E. coli in Nutrient Broth (NB)

For evaluating Str mutant sant agonistic activity against of E. coli O157:H7, the pathogen overnight culture was diluted in 200 mL of fresh NB to obtain cell concentration of approximately 10^5 CFU/mL, divided in two equal portions and supplied LAB in ratios 1:100 and 1:10. Both samples stored at 5 °C and subjected to microbial analysis on days 0, 1, 3, and 5.

2.10 Agar Based Solid State Cocultivation of LAB with Pathogen

Cells from overnight cultures were harvested by centrifugation, washed twice in physiological saline, impregnated by paper disks and placed on E. coli O157:H7 lawn on T-agar, kept for 24 hours at 5 °C then transferred at 37 °C and the next day inhibitory zones around disks were examined.

2.11 LAB Antimicrobial Activity Determination in Ground Meat

150 g of fresh ground beef was inoculated with E. coli O157:H7 to obtain a pathogen concentration of approximately 10^5 CFU/g and divided into three equal portions. LAB cultures were prepared as described previously and at final concentrations of 10^7 CFU/gadded to ground beef samples infected with E. coli O157:H7. The control portions of the ground meat with E. coli O157:H7 were processed in the same manner. All samples were mixed, packaged in vacuum polyethylene packets, kept at 5 °C and subjected for microbiological analysis on days 0, 1, 3, 5 and 7.

2.12 Statistical Analysis

Statistical analysis was performed using SPSS program (Version 16). Standard deviation of mean was used to describe data. Fisher’s range test was used to determine differences between tested groups. P value < 0.05 and 0.001 were considered as significant and highly significant, respectively.

3. Results and Discussion

3.1 UV Mutagenesis and Str Mutants Obtaining

Earlier we have shown that the maximum yield of UV induced resistant to antibiotics mutants in LAB
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occurs at survival about 0.1% [22]. For maximum yield of Str mutants L. delbrueckii cells suspension was irradiated by UV light for 20 sec giving survival ~ 0.1% and plated on T-agar containing 100 µg/ml streptomycin. The UV induced yield of Str mutants was 3.2 ± 0.04 ppm which about 10-20 folds higher than of spontaneous yields.

3.2 Total Selection of H2O2 Active Producers among Str Mutants of L. delbrueckii MH-10

The dominant inhibitory factor produced by lactobacilli at refrigerating temperatures was identified as hydrogen peroxide [14, 23-25]. For full expression of LABs hydrogen peroxide production activity was used modified LAPT g medium because included in its composition yeast extract possess peroxidase activity and high concentration of glucose inhibits hydrogen peroxide formation by LAB [26, 27]. Five strains were isolated in result of testing overnight cultures of Str mutants grown in modified LAPTg broth expecting for active H2O2 formation by Merckoquant Peroxide Test strips.

The selected mutants cells were examined also for production of antimicrobial substances in agar based solid-state condition. Inhibitory zones on pathogen E. coli O157:H7 lawn around disks impregnated by washed cells of Str mutants presented in Fig. 1.

The larger inhibitory zones formed around disks impregnated by cells of DS-105, DS-136 and DS-181 mutants. The storage in refrigerator more than one day was not significantly reflected on the size of inhibitory zones. For one-day solid-state cultivation LAB cells produced as much antimicrobial substances as in submerged cultivation for about three days, probably due to stimulation of hydrogen peroxide production by available oxygen.

3.3 Hydrogen Peroxide Production in BB by L. Delbrueckii Str Mutants during Storage at 5 °C

Three mutants forming larger inhibitory zones were investigated for hydrogen peroxide formation in beef broth. In laboratory experiments for evaluation of hydrogen peroxide production by LAB cells at 5 °C usually was used sodium phosphate buffer [1, 28]. In order to estimate mutant strains potential ability in hydrogen peroxide formation in this study we used beef broth which composition is consimilar with ground beef. The hydrogen peroxide accumulation in BB gradually increased and after five days of cold storage riches maximum in case of all tested strains (Fig. 2). Some of Str mutants about two-fold exceeds the wild strain L. delbrueckii MH-10 in hydrogen peroxide production. The most active mutant DS-181 produces up to 60 µg/mL H2O2.

3.4 Cells Viability and pH Changes in LAB Cultures during Cold Storage

During the entire period of storage at 5 °C live cells count and beef broth cultures pH were monitored (Table 1).

No significant differences were found in the population levels of LAB cultures during over 7 days storage at 5 °C indicating that LAB reproduction was
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Fig. 2 Hydrogen peroxide production by *L. delbrueckii* and Str mutants at 5 °C in beef broth.

Table 1 LAB viability and pH in BB during storage at 5 °C.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Day zero pH</th>
<th>Viable cells, CFU/mL</th>
<th>After 7 days pH</th>
<th>Viable cells, CFU/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. delbrueckii</em> MH-10</td>
<td>6.5 ± 0.2</td>
<td>6.3 × 10^8</td>
<td>6.4 ± 0.2</td>
<td>6.2 × 10^8</td>
</tr>
<tr>
<td>DS-105</td>
<td>6.7 ± 0.2</td>
<td>6.7 × 10^8</td>
<td>6.5 ± 0.2</td>
<td>5.1 × 10^8</td>
</tr>
<tr>
<td>DS-136</td>
<td>6.8 ± 0.2</td>
<td>7.5 × 10^8</td>
<td>6.3 ± 0.2</td>
<td>7.6 × 10^8</td>
</tr>
<tr>
<td>DS-181</td>
<td>6.8 ± 0.2</td>
<td>8.3 × 10^8</td>
<td>6.1 ± 0.2</td>
<td>8.6 × 10^8</td>
</tr>
</tbody>
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Fig. 3 *E. coli* O157:H7 inactivation by LABs during submerged co-cultivation in NB at 5 °C by (∗)—*L. delbrueckii* MH-10; (■)—Str mutant DS-181; (▲)—*E. coli* O157:H7 control.

not necessary for hydrogen peroxide formation. These findings come in accordance with the observations of Amezquita and Brashears 2002, Ruby and Ingham 2009 who suggested that the production of inhibitory metabolites can occur by LAB during storage in the absence of growth [1, 28].

3.5 Antagonistic Action of LABs on *E. coli* O157:H7 in submerged Cocultivation at 5 °C

*L. delbrueckii* and Str mutant DS-181 were added to NB broth along with *E. Coli* O157:H7 in ratio 1:100 in order to determine their antagonistic action against the pathogen at 5 °C. The total number of *E. coli* O157:H7 cells in both treatments were determined on days 0, 1, 3 and 5 by plating appropriate dilutions on Tryptose agar and incubation at 37 °C for 24 hours.

The parental and mutant strains of *L. delbrueckii* MH-10 significantly reduced the initial populations of *E. coli* O157:H7 (Fig. 3). For 3 days of storage, the mutant DS-181 practically completely eliminates *E. coli* O157:H7 whereas the wild strain declines the number of pathogen for 2 log. The number of viable cells of *E. coli* O157:H7 in control sample was not significantly changed during storage in
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non-permissive temperature. These results are similar with date obtained by Brashears et al. [29].

3.6 LABs Inhibitory Effect on E. coli O157:H7 in Ground Meat at 5 °C

L. delbrueckii MH-10 and Str mutant DS-181 were tested in packaged ground meat for their ability to reduce the viability of Escherichia coli O157:H7 during storage at 5 °C in plastic vacuum bags. Each ground meat sample was infected with 10^5 CFU/g of E. coli O157:H7 and treated with 10^7 CFU/g of LABs. Samples were analyzed for E. coli O157:H7 survivors and lactic acid on days 1, 3, 5 and 7 (Fig. 4).

Towards the end of ground meat vacuum storage E. coli O157:H7 quantity was 3-4 log lower than those in the control. The impact of Str mutant on the pathogen was significantly higher of L. delbrueckii MH-10.

Growth of LABs in a fresh meat held at refrigeration temperature is not desirable because it would lead to premature spoilage of the product. The count LABs in treated samples for 7 days were not significantly changed (date was not shown). It was revealed that the application of Str mutant has not any influence on sensory characteristics of ground beef, moreover promote expanding of shelf-life due to inhibition of psychrophilic spoilage microorganisms which is in agreement with other authors Gilland et al. [30] and Senne and Gilland [31].

Experiments suggest that treated by Str mutant ground beef keeps good quality for entire period of storage; avoid appearance of undesirable odor, greening and smooth on meat surface by synthesizing antimicrobial compounds in amounts sufficient to inhibit the growth of pathogens and spoilages. Thus, Str mutations have high potential to be used for improvement antimicrobial compounds production LABs intendent for biopreservation meat products in cold storage.

The fundamental connections between streptomycin resistance, ribosome structure and function and global gene expression are not doubt. It has been shown that a variety of Escherichia coli mutants, deficient in different metabolic functions, are able to grow in presence of streptomycin even in absence of specific growth factors [32, 33]. It is known, that resistance to streptomycin is associated with mutations in the gene coding for the S12 protein of (rpsL) the small ribosomal subunit, which reads the information in mRNA molecules [33]. Bacterial mutants selected for their resistance to streptomycin frequently possesses high pleiotropy: partially suppress the temperature-sensitivity; reduce the rate of growth; suppress the restriction imposed by prophage PI to the growth of phage previously grown on a strain non lysogenic for PI [34, 35]. Str mutations of ribosome have been found involved in a variety of physiological processes and possessing pleiotropic effects, including: cell growth [32, 36]; the ability to interact with other genes mutant alleles; thermosensitivity and intrinsically resistance of LABs to antibiotics [19-37].

It was shown that hydrogen peroxide producing ability of LAB at non permissive temperature is strongly dependent on nutrition media composition used for their prior propagation as well as media for sub cultivation at refrigeration temperatures. For largest amount of hydrogen peroxide production LAB cells must be priory propagated in rich medium and

![Fig. 4 Kinetic of E. coli O157:H7 inactivation by L. delbrueckii MH 10 and Str mutant in ground meat at 5 °C. (■)—E. coli O157:H7 + DS-181; (▲)—E. coli O157:H7 + L. delbrueckii MH 10; (♦)—E. coli O157:H7 control.](image-url)
then transferred in sodium phosphate buffer at 5 °C [1, 28]. Cell suspensions in phosphate buffer without glucose showed high accumulation of H2O2 in contrast to phosphate buffer containing glucose where produced undetectable amounts of H2O2. High concentrations of glucose appeared to inhibit the production of H2O2 by the cells [24, 26, 38]. In our experiments at the first time we used beef powder broth very close to the ground beef as model for evaluation H2O2 production and found yield higher than in phosphate buffer.

4. Conclusion

Resistant to streptomycin mutants of L. delbrueckii MH-10 produced significant amounts of hydrogen peroxide at 5 °C in beef broth without of growth. They exert higher inhibitory action against E. coli O157:H7 and practically fully eliminate the pathogen bacteria from ground beef at refrigeration temperatures. Str mutations can be successfully used as a tool in biotechnology for improving preservative properties of LABs intended for commercial applications.

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References


