Mutational Improvement of *Lactobacillus acidophilus GH 201* Intended for Ground Beef Preservation in Refrigerator Storage

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**Abstract:** Lactic acid bacteria widely used in food preservation at refrigerator temperatures due to their ability produce high amount of hydrogen peroxide and/or other antibacterial substances at refrigerator temperatures to inhibit food-borne pathogens and psychrophilic spoilage microorganisms. In order to improve of bio-preservation efficacy of *Lactobacillus acidophilus GH 201* mutations causing resistance to rifampicin (*rif*) were used. Among UV-mutagenized population of *L. acidophilus* five *Rif* mutants producing high amounts of H$_2$O$_2$ were selected. *Rif* mutants produced significant amounts of hydrogen peroxide 50-55 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. The mutants possess higher impact against food-borne pathogen *Escherichia coli* O157: H7 at refrigeration temperatures and for 3 days reduces the pathogen total amount practically undetectable level. *Rif* mutants *L. acidophilus* reduced initial amount 2x10$^5$ 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. acidophilus GH 201* mutants did not cause any changes in sensory characteristics of ground beef, moreover promotes expanding of shelf-life due to inhibition of psychophilic spoilage microorganisms.

**Keywords:** Biopreservation, *Lactobacillus acidophilus*, *rif* Mutation, Refrigerator 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 GRAS (Generally Recognized As Safe) 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 these products [6, 11, 12]. The species *L. delbrueckii* subsp. lactis more of all used in food
preservation, but hydrogen peroxide production is variable amongst strains [13, 14]. 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 [15-17]. Because hydrogen peroxide production plays the major role in elimination of the pathogens at refrigerator storage, it levels should be assessed for newly selected strains. There aren’t 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 remains great challenge 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 dairy starters and other industrial microorganisms [18, 19].

There are fundamental connections between rifampicin resistance, RNA polymerase structure and function and global gene expression. Resistance to rifampicin is associated with mutations in the gene coding for the beta subunit of RNA polymerase (rpoB) [20, 21]. Rif mutations of RNA polymerase have been found involved in a variety of physiological processes and possessing pleiotropic effects, including: cell growth [22-24]; 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 [22]; exopolysaccharides oversynthesis and thermosensitivity of LABs [18, 19]. A spontaneous rif mutation of Saccharopolyspora erythraea caused slow-growth and stimulated erythromycin production [25].

The objective of this study selection of rif mutants L. acidophilus GH 201 possessing high hydrogen peroxide production ability and evaluation their impact on E. coli O157:H7 in ground beef during refrigerated storage.

2.3. Mutagenesis and Selection of Rifampicin Resistant Mutants

L. acidophilus GH 201 cells grown at 37°C in MRS (OD 0.6) were harvested by centrifugation at 12,000 x g for 15 min and resuspended 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 MRS broth and were grown at 37°C for 4 h to permit 3 - 4 division cycles. For obtaining rif mutants cells were plated on MRS agar containing 100 µg/ml of rifampicin and incubated at 37°C till resistant colonies appearance.

2.4. Bacteriological Analysis

Bacterial count in liquid media was made using standard methods [26]. 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. 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 0.1 ml 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.

2.7. Quantification of Antimicrobial Activity of LAB Cultures in Cold Cultivation

Lactobacilli were grown in MRS broth for 18 h at 37°C divided in four 10 ml aliquots, centrifuged at 12,000 x 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.

2.8. Submerged Cocultivation of LAB and E. coli in Nutrient Broth (NB)

For evaluating rif mutants antagonistic 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 10^3 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, 5, and 7.
2.9. 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 inhibitory zones around disks were examined on the next day.

2.10. 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/ml added into two samples of infected ground beef mixed and packaged in vacuum polyethylene bags. The control portions of the ground meat with *E. coli* O157:H7 were processed in the same manner. All samples were kept at 5°C and subjected for microbiological analysis on days 0, 1, 3, 5 and 7.

2.11. 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 rif Mutants Obtaining

Earlier we have shown that the maximum yield of UV induced resistant to antibiotics mutants in LABs occurs at survival about 0.1% [27]. For maximum yield of rif mutants *L. acidophilus* GH 201 cells suspension was irradiated by UV light for 20 sec giving survival ~ 0.1 % and plated on T-agar containing 100 µg/ml rifampicin. The UV induced yield of rif mutants was 3.2 ± 04 ppm which about 10 – 20 folds higher than of spontaneous yields.

3.2. Total Selection of H_2O_2 Active Producers Among of *L. Acidophilus* GH 201 rif Mutants

The dominant inhibitory factor produced by lactobacilli at refrigerating temperatures was identified as hydrogen peroxide [13, 28-30].

Among tested overnight cultures of 300 rif mutants grown in MRS broth by Merckoquant Peroxide Test strips, four mutants expecting for active H_2O_2 formation were isolated. The mutants were examined for production of antimicrobial substances at 5°C by Tryptose agar based solid-state cultivation on *E. coli* O157:H7 lawn. The growth inhibitory zones of pathogen around disks impregnated by washed cells of rif mutants presented in Figure 1.

3.3. Hydrogen Peroxide Production in BB by *L. Acidophilus* GH 201 rif 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, 31]. 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. Beside this, for full expression of LABs hydrogen peroxide production activity, the MRS is
not satisfy because include yeast extract and high concentration of glucose which possesses respectively peroxidase and hydrogen peroxide formation inhibitory activities [32, 33]. 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). Rif mutants by hydrogen peroxide production 70-80 % exceeds parental strain L. acidophilus GH 201. The most active mutant LR-195 produces $H_2O_2$ mean 55 µg/ml.

### 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 culture pH were monitored (Table.1).

| Strain                  | Day zero pH | Viable cells, CFU/ml | After 7 days pH | Viable cells, CFU/ml |
|-------------------------|-------------|----------------------|-----------------|----------------------|
| L. acidophilus GH 201   | 6.5 ± 0.2   | 7.6 x 10^8           | 6.4 ± 0.2       | 7.2 x 10^8           |
| LR-128                  | 6.7 ± 0.2   | 7.8 x 10^8           | 6.5 ± 0.2       | 7.7 x 10^8           |
| LR-195                  | 6.8 ± 0.2   | 8.5 x 10^6           | 6.5 ± 0.2       | 8.6 x 10^6           |
| LR-247                  | 6.8 ± 0.2   | 8.3 x 10^6           | 6.6 ± 0.2       | 8.5 x 10^6           |
| LR-286                  | 6.8 ± 0.2   | 8.4 x 10^6           | 6.6 ± 0.2       | 8.6 x 10^6           |

No significant differences were found in the population levels of LAB cultures during over 7 days storage at 5°C indicating that cells reproduction was 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, 31].

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

L. acidophilus GH 201 and most active rif mutant LR-195 about $10^7$ CFU/ml were separately added into NB broth along with E. coli O157:H7 $10^5$ CFU/ml in order to determine their comparative 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 impact of mutant and parental strains of L. acidophilus GH 201 on E. coli O157:H7 in nutrition broth significantly differ (Figure. 3). For 3 days of storage, the mutant LR-195 reduces the initial populations of E. coli O157:H7 ~ 3.5 log whereas the wild strain only 2.3 log. In the control sample viable cell number of E. coli O157:H7 was not significantly changed for 7 days.

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

L. acidophilus GH 201 and rif mutant LR-195 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.

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 rif mutant on the pathogen was significantly higher of L. acidophilus GH 201.

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 wasn't significantly changed. It was revealed that the application of rif mutant hasn't 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 [6, 34-36]. Experiments suggest that treated by rif 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, rif mutations have high potential to be used for improvement antimicrobial compounds production LABs intend for biopreservation meat products in cold storage.

It was shown that hydrogen peroxide production ability of LAB at 5°C 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 priory propagated in rich medium and then transferred in sodium phosphate buffer at 5°C [1, 32]. 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 [29, 32, 37]. In our experiments at the first time we used beef powder broth very close to the ground beef by composition as model for evaluation H2O2 production and found yield significantly higher than in phosphate buffer.

A characteristic feature of rif mutations is pleiotropy of their phenotypic realization. They are able to cause misreading of genetic information at the level of transcription over whole genome that brings to simultaneous alterations of large spectrum phenotype features of bacteria e. g. colony morphology, growth rate, sensitivity to temperature, requirements in growth factors, production of secondary metabolites etc., by global changing of transcriptional profile [38-43]. Thus, it was possible to show that mutations deter mining resistance to rifampicin may be an efficient instrument for selecting strains of lactobacilli with enhanced yield of antimicrobial substances including hydrogen peroxide.

4. Conclusion

Resistant to rifampicin mutants of mutants L. acidophilus GH 201 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. Rif mutations can be successfully used as a tools in biotechnology for improving preservative properties of LABs intended for commercial applications.

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