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Antimicrobial Substances Production at Refrigeration Temperatures by *Lactobacillus delbrueckii* MH10: A Candidate for Food Biopreservation

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Abstract: The objective of this study was to determine formation of antibacterial substances in supernatants of *L. delbrueckii* during cold storage and evaluate whether the application of this bacteria to raw ground beef would result in significant reductions of *E. coli* O157:H7 during refrigerated storage. Antibacterial activity of a newly isolated *Lactobacillus delbrueckii* MH 10 at refrigeration temperatures against food-borne pathogen *Escherichia coli* O157: H7 was studied. The size of inhibition zone depends on concentration of LAB cells. The cells (~10^9 CFU/ml) of *L. delbrueckii* produced significant amount of antibacterial substances mainly hydrogen peroxide ~ 35 µg/ml in sodium phosphate buffer (0.2 M, pH 6.5) and ~ 40 µg/ml in beef broth at 5°C during submerged cultivation without of growth. Submerged cocultivation of *E. coli* O157: H7 with lactobacilli in NB broth at 5°C reducing the total number of the pathogen more than 3 log for 5 days. The cell suspension intendent for treatment must contain 10^{8-9} CFU/ ml of LAB. *L. delbrueckii* reduced initial amount 2x10^5 of *E. coli* O157: H7 in ground beef cocultivation up to 3 log for 3 days and become undetectable after 7 days. The application of *L. delbrueckii* bacteria does not cause any changes in sensory characteristics of ground beef by itself; moreover promote expanding of shelf-life due to inhibition of psychrophilic spoilage microorganisms.

Keywords: Biopreservation, *Lactobacillus delbrueckii*, 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]. For these applications lactic acid bacteria are usually chosen as they have the GRAS (Generally Recognized As Safe) status and 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 [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].

The species *Lactobacillus delbrueckii* subsp. lactis more of all used in food preservation, but hydrogen peroxide production is variable amongst strains [11, 12]. 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 [2]. In order to enhance biopreservation efficacy new LABs producing hydrogen

peroxide should be selected and methods developed for their cultivation and application to food [13-15]. Because hydrogen peroxide production plays the mayor role in elimination of the pathogens at refrigerator storage, it levels should be assessed for newly selected strains.

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. Although many intervention technologies are applied to beef carcasses, ground beef processors currently do not have effective intervention steps for ground beef safe storage. There are only a few studies of LAB inhibition of *E. coli* O157:H7 in ground beef products and their impact on the sensory properties of these products [6, 16, 17].

The aim of this research was to determine formation of antibacterial substances supernatants of *L. delbrueckii* during cold storage and evaluate whether the application of this bacteria to raw ground beef would result in significant reductions of *E. coli* O157:H7 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 9617. Food born pathogen *Escherichia coli* O157: H7 MDC 5003 used in this study was from the MDC.

2.2. Media

LAPTg: yeast extract – 10 g, peptone -15 g, tryptone – 10 g, glucose – 10 g, Tween -1ml, for solid medium, 1.5% Bacto-agar was included. Nutrient broth (NB) [Serva, Germany], Tryptose agar (T-agar) [Merck, Germany]. beef broth: Beef extract powder (Sigma Aldrich) – 30 g to 1000 ml of distilled water, pH 6.0 - 7.0. Fresh ground beef were 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. Bacteriological Analysis

Bacterial counts in liquid media were made using standard methods. For enumeration of *E. coli* and lactobacilli in ground beef 1 g treated 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.

2.4. 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.

2.5. 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.

2.6. 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 spread onto Tryptose agar. The paper discs (diameter, 5 mm) were soaked with LAB culture liquids and placed on the test culture lawn. After 2h 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 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 (with or without glucose), LAPTg broth or physiological saline 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 Along E. Coli in Nutrient Broth (NB)

For evaluating the antagonistic activity of *L. delbrueckii* MH 10 against of *E. coli* O157:H7 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 *L. delbrueckii* MH 10 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. LAB Antimicrobial Activity Determination in Ground Meat

200 g of freshly prepared commercial ground beef was obtaining from local grocery. 150 g of this ground beef was inoculated with *E. coli* O157 to obtain a pathogen concentration of approximately 10^5 CFU/g and divided into three equal portions. *L. delbrueckii* MH 10 was prepared as described previously and added individually in two of ground beef samples inoculated with *E. coli* O157:H7 at final concentrations of 10^7 and 10^8 CFU/g respectively. The control portions of the ground meat with and without *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, 7 and 10.

2.10. Sensory Evaluation

Sensory evaluation of the control and the samples inoculated with the LAB strains was conducted in the open laboratory. Odor, color and appearance of slime on the external surface of ground meat were assessed.

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. Antibacterial Substances Production by Washed L. Delbrueckii Cells at 5°C

Laboratory experiments revealed 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. The largest amount of hydrogen peroxide at 5°C formed when LAB cells priory propagated in rich medium and then transferred in sodium phosphate buffer [1, 18]. In this study along with phosphate buffer we used beef broth in order to ensure the lack of substances inhibitory to antimicrobials formation e. g. hydrogen peroxide.

Antimicrobial compounds production by 2 fold dilutions of washed cells *L. delbrueckii* MH 10 in beef broth and phosphate buffer were studied during storage at 5°C by disk diffusion method (Figures 1, 2).

The growth inhibition zones of *E. coli* O157:H7 around the disks, impregnated in the both media, gradually increased for 5 days period in cold storage.

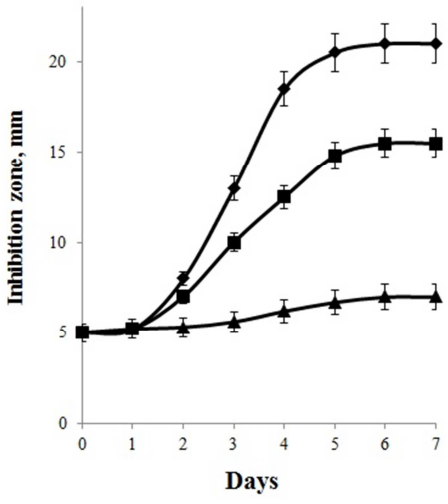

Figure 1. *Zones of E. coli O157: H7 growth inhibition caused by supernatants of different concentrations L. delbrueckii washed cells inoculated in meat extract at 5°C. Cell concentration CFU/ml (♦) 2 x 10^8, (■) 6 x 10^7, (▲) 3 x 10^7.*

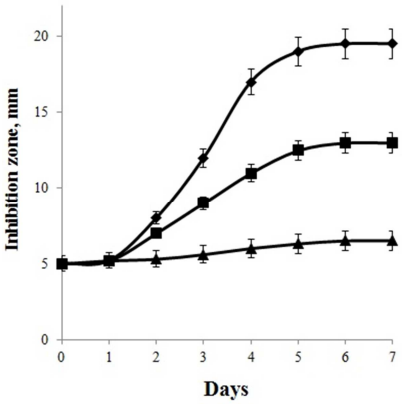

Figure 2. *Zones of E. coli O157: H7 growth inhibition caused by supernatants of different concentrations L. delbrueckii washed cells inoculated in Phosphate buffer at 5°C. Cell concentration CFU/ml (♦) 2 x 10^8, (■) 6 x 10^7, (▲) 3 x 10^7.*

Inhibitory compounds formation in beef broth by *L. delbrueckii* at refrigerating temperatures was shown for the first time. The inhibitory zones caused by cells inoculated in beef broth were remarkably larger than in phosphate buffer. The inhibitory zones size required high concentration of cells.

3.2. Hydrogen Peroxide Production by L. delbrueckii During Storage at 5°C

The dominant inhibitory factor produced by lactobacilli at refrigerating temperatures was identified as hydrogen peroxide [11, 19-21]. In this study production of hydrogen peroxide by *L. delbrueckii* at 5°C storage were obtained. The hydrogen peroxide accumulation by lactobacilli in both media phosphate buffer and beef broth gradually increased and riche maximum after five days of cold storage. Cell suspensions in beef broth showed higher accumulation of H_2O_2 in comparison to phosphate buffer. The overall H_2O_2 amount in phosphate buffer and beef broth were approximately 35 and 40μg/ml, respectively (Fig. 3, 4). Thus, the strain MH 10 by hydrogen peroxide production was not inferior to known strains *L. delbrueckii* [20, 22].

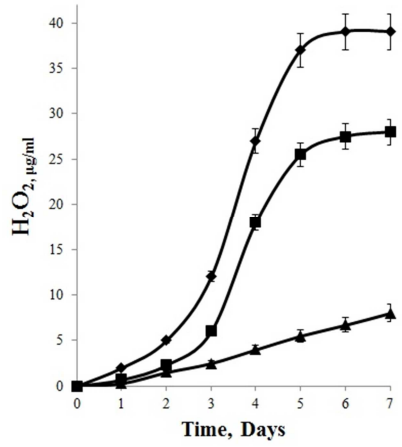

Figure 3. *Kinetic of hydrogen peroxide production by L. delbrueckii MDC 9617 at 5°C in beef broth. Cell concentration CFU/ml (♦) 2 x 10^8, (■) 6 x 10^7, (▲) 3 x 10^7.*

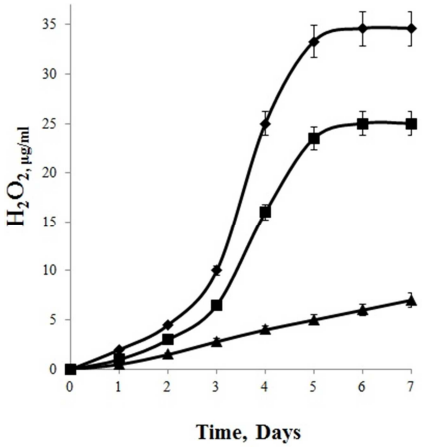

Figure 4. *Kinetic of hydrogen peroxide production by L. delbrueckii MDC 9617 at 5°C in phosphate buffer. Cell concentration CFU/ml (♦) 2 x 10^8, (■) 6 x 10^7, (▲) 3 x 10^7.*

It was revealed that the character of hydrogen peroxide accumulation was similar to antibacterial activity growth in LAB suspension (see figures 1, 2) which indicates on main role of hydrogen peroxide in inhibitory action lactobacilli against *E. coli* O157: H7 at refrigerator storage.

3.3. Cells Viability and Acidity Changes During Cold Storage

During the entire period of storage at 5°C live cells count and culture pH and titrable acidity were monitored (Table 1).

Table 1. *Cells viability and acidity in the media during storage at 5°C.*

	Day zero		After 7 days	
Medium	**pH**	**Viable cells, CFU/ml**	**pH**	**Viable cells, CFU/ml**
Beef broth	6.7±0.2	6.3±0.3 x 10^8	6.5±0.2	5.8±0.4 x 10^8
PBS	6.8±0.05	6.3±0.2 x 10^8	6.8±0.05	5.6±0.2 x 10^8

Footnote: Titrable acidity i.e. lactic acid in samples was not detected.

No significant differences were found in the population levels of LAB cultures during entire storage period at 5°C indicating that LAB reproduction was not necessary for the inhibition of pathogens. These findings come in accordance with the observations of other authors who also suggested that the production of inhibitory metabolites can occur by LAB during storage in the absence of growth [1, 18]. Any significant changes of pH and titrable acidity in all of media weren't detected which indicated that the organic acids are not formed.

3.4. Antagonistic Action of L. delbrueckii on E. coli O157:H7 in NB at 5°C

L. delbrueckii was added to NB along with *E. coli* O157:H7 in order to determine its antagonistic activity against the pathogen at 5°C. Two ratios of *E. coli* O157:H7 to *L. delbrueckii* 1: 100 and 1: 10 were tested. The total number of *E. coli* O157:H7 cells in both treatments were determined on days 0, 1, 3, 5 and 7 by plating appropriate dilutions on Tryptose agar and incubation at 37°C for 24 hours.

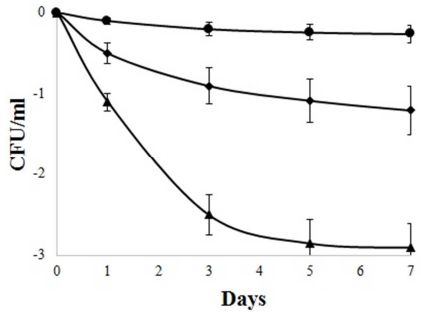

Figure 5. *Kinetic of inactivation of E coli O157:H7 by L. delbrueckii in NB submerged cocultivation and in at 5°C.*

(■) – *E. coli* O157:H7, (♦) *E coli* O157:H7 + L. *delbrueckii* MH 10 ratio (1:10), (▲) – *E coli* O157:H7 + L. *delbrueckii* MH 10 ratio (1:100).

On day zero there were no significant differences among the initial populations of *E. coli* O157:H7 for all treatments (Figure. 5). After 3 days of storage, there were significant decline in numbers of *E. coli* O157:H7 for treatments containing 1.1 x 10^8 of L. *delbrueckii*. There was an additional significant decline (3 log) in the numbers of viable cells of *E. coli* after 5 days of storage. There was only 1 log decline in the treatment containing 1.4 x 10^7 CFU/ml of lactobacilli. It suggesting that, there sufficient numbers of LAB must be present to have an antagonistic effect on pathogens. The number of viable cells of *E. coli* O157:H7 in control was not significantly changed in non-permissive temperature. These results are similar with date obtained by Brashears et al [23].

3.5. LAB Inhibitory Effect on E. coli O157:H7 in Ground Meet at 5°C

Cell suspension of *L. delbrueckii* was tested in packaged ground meat stored for their ability to reduce the viability of *Escherichia coli* O157:H7 during storage temperature at 5°C. Fresh ground meat was inoculated with 10^5 CFU/g of *E. coli*. The trial samples were treated with *L. delbrueckii*, at a level of 10^7 and 10^8 CFU/g and stored at 5°C for 7 days in plastic vacuum bags. Samples were analyzed for *E. coli* O157:H7 survivors and lactic acid bacteria on days 1 to 7.

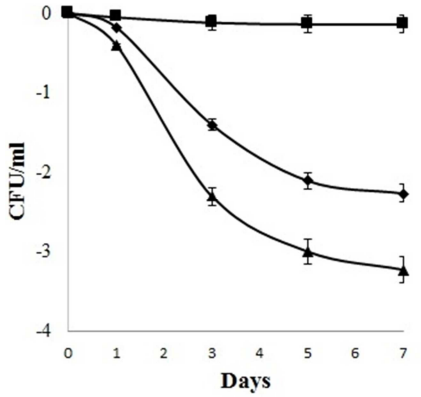

Figure 6. *Kinetic of E. coli O157:H7 inactivation by L. delbrueckii MH 10 in ground meat at 5°C. (■) – E. coli O157:H7, (♦) E coli O157:H7 + 10^7 L. delbrueckii MH 10, (▲) – E coli O157:H7 + 10^8 L. delbrueckii MH 10.*

Towards the end of ground meat vacuum storage *E. coli* O157:H7 quantity, depending of *L. delbrueckii* ratio, was 2-3 log lower than those in the control. But the LAB count in treated and control samples after refrigeration storage didn't significantly change for 10 days. Growth of LAB in a fresh meat held at refrigeration temperature is not desirable because it would lead to premature spoilage of the product. Furthermore, the viable *L. delbrueckii* cells in ground beef, continuous protection by preventing secondary contamination during entire storage period.

It was revealed that the application of *L. delbrueckii* bacteria doesn't causes any changes in sensory characteristics of ground beef entire 10 days storage, moreover promote expanding of shelf-life due to inhibition of psychrophilic spoilage microorganisms which is in agreement with other authors [6, 24-27]. Thus, the LAB treated samples keep good quality for ten days whilst in the control sample at 7 day of storage appeared undesirable odor, greening and smooth on surface. Therefor the selected strain *L. delbrueckii* MH 10 is able to synthesize antimicrobial compounds in amounts sufficient to inhibit the growth of pathogens and spoilages.

The results of the experiments suggest that *L. delbrueckii* MH 10 has a potential to be used as a candidate culture in biopreservation method to improve the safety and extend the shelf life of meat products in cold storage.

4. Conclusion

The cells of *L. delbrueckii* MDC 9617 produced significant amount of antibacterial compounds mainly hydrogen peroxide in beef broth and sodium phosphate buffer at 5°C in absence of growth. This strain exerts inhibitory action against *E. coli* O157:H7 and dramatically (more than 95%) reduced number of pathogen bacteria in both submerged and solid-stat cocultivation trials at refrigeration temperatures. Thus, *L. delbrueckii* can be recommended as candidate bio-preservative for use in commercial applications.

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