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Consideration of Lactic Acid Bacteria Ability to Reduce Nitrite Concentration in Standard De Man, Rogosa and Sharpe (Mrs) Broth-Sodium Nitrite Medium During Fermentation Period

[1]Peiman Esmaeilzadeh, [1]Shole Darvishi, [2]Keiwan Ebrahimi, [1]Fardin Mirahmadi and [1]Moharam Vaziri

[1]Department of Food Science and Technology, Faculty of Agriculture,
Islamic Azad University, Sanandaj Branch, Sanandaj, Iran
[2]Department of Food Science and Technology, Faculty of Agriculture,
Islamic Azad University, Mahabad Branch, Mahabad, Iran

Abstract: Nitrite is still considered as basically undesired in foods so controlling their concentration is important in standpoint of food safety. In the present study, selected lactic acid bacteria (LAB) obtained from Persian Type Culture Collection (PTCC) and the ability of these bacteria to deplete nitrite during fermentation period in standard de Man, Rogosa and Sharpe (MRS) broth-sodium nitrite, including 120µg/ml Sodium nitrite was determined. Pure *Lactobacillus plantarum* PTCC 1058*, Lactobacillus fermentum* PTCC 1638*, Leuconostoc mesentroides* subsp *mesentroides* PTCC 1563 and mixed LAB namely, *L. plantarum* and *L. fermentum*, *L. plantarum* and *Leu. mesentroides*, *L. fermentum* and *Leu. mesentroides* mixtures (as starter culture) with cell density of approximately 9×10^8cfu/ml were prepared and inoculated to standard MRS broth-sodium nitrite solution. The results demonstrated that all the strains depleted nitrite concentration but Pure and mixed *Lactobacilli* strains reduced nitrite concentration significantly ($P<0.05$). Hence, ability of *L. plantarum* and *L. fermentum* to deplete nitrite could be the reason for more researches about their ability in real food media.

Key words: MRS broth · Lactic acid bacteria · Nitrite reduction · Fermentation period

INTRODUCTION

Sausages-fermented or heated-are very popular meaty products in the world, they can be manufactured either traditionally or commercially [1]. Consumption of these products as fast foods is expanding in Iran like other progressive countries. A critical point is nitrite as additives used to sausages formulation. Nitrite causes some special properties such as color, flavor, prevention of oxidation, helping to domination of gram-positive bacteria, stability and hygienic safety [2-5]. Excess vegetables such as cabbage, cucumber, celery and radish are normally preserved into pickles or other fermented vegetables also fermented vegetables are traditional dish favored in Iran. Those are normally served as a side dish with the main meal or used as an appetizer. Cammarical vegetable fermentation are normally based on spontaneous fermentation that is highly depended on the epiphytic microbe present on the raw materials [6]. In such as products an accumulation of nitrite is a common problem faced during vegetable fermentations [7]. During fermentation nitrate present in the planet tissue is reduced to nitrite [8]. On the other hand, nitrite recognized as tratogenic and carcinogenic substance because of extreme reductive and oxidative activities [9,5]. Moreover, Consumption of nitrite has been linked to methemoglobinemia and incidence of cancers. Methemoglobinemia is a condition where reduced iron (Fe^{2+}) in haemoglobin is oxidized by nitrite to it's maximum oxidized state (Fe^{3+}), thus reducing the total oxygen carrying capacity of blood [10]. Extensive experimental and epidemiological data suggest that human are susceptible to carcinogenesis N-nitroso compounds resulted from endogenous nitrosatoin reaction of nitrite [11]. There have been many attempts to reduce the N-nitroso compound concentration in fermented foods. Some lactic acid bacteria are found to contribute to the depletion of nitrite in m any foods [8,12,13]. Whereas,

Corresponding Author: P. Esmaeilzadeh, Department of Food science and Technology,
Faculty of Agriculture, Islamic Azad University, Sanandaj Branch, Sanandaj, Iran.
Tel: +98-9188752386.

lactic acid bacteria play main rolls in fermented vegetables and sausages usage of starter cultures with selected lactic acid bacteria is very effective for nitrite reduction. In the most studies, the determination of LAB's nitrite reduction abilities were carried out in MRS broth then were considerated in real or simulated foods media [8,14-16]. In the present study, we investigated some pure lactic acid bacteria (Heterofermentative - Homofermentative) and their mixtures capability for depletion of nitrite concentration in MRS broth during fermentation period. The microorganisms were selected according to the frequency of occurrence in fermented sausage and vegetable products.

MATERIAL AND METHODS

Material: The lactic acid bacteria strains were obtained from Persian Type Culture Collection (PTCC), Iranian Research Organization for Science and Technology (IROST), included *Lactobacillus fermentum* PTCC 1638, *Lactobacillus plantarum* PTCC 1058 and *Leuconostoc mesentroides* subsp *mesentroides* PTCC 1563 existed in microbial bank officially in lyophilized forms. They were isolated from fermented olive.

Strain Activation: LAB were activated as described by instruction of PTCC. Lyophilized tubes revived aseptically, the tubes with *L. plantarum*, *L. fermentum* and control tube were incubated at 37?, *Leu. mesentroides* and control tube were incubated at 30? in anaerobic conditions in the BBL Gas Pak Plus Anaerobic System (Beckon Dickinson Microbiology System, Cockeysville, MD, USA) for 80 h. Each tube was cultured with Spread Plat Method (SPM) on MRS agar (Merck, Germany) and incubated in respective incubation conditions for 42-72 h with control plates [17]. The tubes were sealed with Parafilm and kept in the refrigerator in anaerobic condition. The LAB colonies were passed alternatively to get strong and single colonies.

Mcfarland Turbidity Standard: McFarland standards used to evaluation visually approximate of concentration of cells in a suspension. The 0.5 McFarland was prepared by adding 0.5 ml of a 1.175% (w/v) barium chloride dihydrate ($BaCl_2 \bullet 2H_2O$) (Merck, Germany) solution to 99.5 ml of 1% (v/v) sulfuric acid (Merck, Germany). The turbidity standard is then aliquoted into test tubes identical to those used to prepare the inoculums suspension [18,19]. The accuracy of the density of a prepared McFarland standard was checked by using a spectrophotometer (Jenway 1653, LTD, UK) with a 1-cm light path, the 0.5 McFarland standard absorbance at a wavelength of 600 nm was 0.134.

Bacterial Suspension Preparation (Starter Culture): Three well-isolated colonies on overnight culture surfaces were harvested with an inoculating loop and transfer the growth to a tube of sterile saline and vortex thoroughly. The bacterial suspensions were compared to the 0.5 McFarland standard, this comparison was viewed against a sheet of white paper on which sharp black lines are drawn. The accuracy of the McFarland standard was verified by adjusting bacterial suspensions, preparing serial 10-fold dilutions then performing plate counts [4,20]. However, three LAB strains selected from PTCC, pure *L. plantarum, L. fermentum*, *Leu. mesentroides* and their mixtures in 1:1 (v/v) ratio namely, *L. plantarum* and *L. fermentum*, *L. plantarum* and *Leu. mesentroides*, *L. fermentum* and *Leu. mesentroides* mixtures were prepared *in vitro* with cell density of approximately 9×10^8 cfu/ml as starter cultures.

Depletion of Sodium Nitrite by LAB: Ability of LAB to deplete sodium nitrite was determined as described by Yan *et al*. One ml of 120µg/ml sterilized sodium nitrite solution was added to 9 ml of MRS broth in test tubes (as standard MRS-sodium nitrite solution). Control tubes were prepared without any inoculums [8]. Six LAB inoculums were inoculated separately into media, tubes with *L. plantarum*, *L. fermentum* and *L. plantarum* and *L. fermentum* mixtures were incubated at 37? for 4 days, tubes with *Leu. mesentroides*, *L. plantarum* and *Leu. mesentroides* and *L. fermentum* and *Leu. mesentroides* mixtures were incubated at 30? for 4 days in the BBL Gas Pak Plus Anaerobic System (Beckon Dickinson Microbiology System, Cockeysville, MD, USA) as fermentation period. The depletion of sodium nitrite was determined by colorimetric nitrite assay measuring on 0,1,2,3 and 4th day of fermentation.

Nitrite Concentration of Standard MRS-Sodium Nitrite Solution: Nitrite concentration was determined using colorimetric nitrite assay as described by Institute of Standards and Industrial Research of Iran NO:923. Approx 10 ml inoculated standard MRS-sodium nitrite solution, defated and deproteinated with 5 ml disodium tetra borate dehydrate (Merck, Germany), 2 ml potassium ferrocyanide trihydrate (Merck, Germany), 2 ml mixture of zinc acetate dehydrate (Merck, Germany) and glacial acetic acid (Merck, Germany), the mixture was kept for 30 min at room

temperature and followed by filtration. 10 and 6 ml of tow color development regents, namely 0.2% sulfanilamide (Merck, Germany) and thick cloridric acid (Merck, Germany) respectively was add sequentially to the filtrates. That was kept for 5 min in dark place at room temperature, then 2 ml of 0.1 % α-Nephtylene diamin (Merck, Germany) and was mixed thoroughly. The mixtures were kept at room temperature for 3-10 min. OD of colored mixtures read at 538 nm against the reagent blank. To construct a standard curve, 0-3000 µg/ml sodium nitrite solutions was subjected to the similar color development and OD measurement steps [21]. The nitrite assay was carried out by using a Jenway spectrophotometer (Jenway 1653, LTD, UK).

Statistical Analysis: Data were analyzed by tow factorial analysis in a completely randomized design. The two factors were: (1) nitrite concentration of MRS broth and (2) fermentation time. Means were compared using Duncan's multiple range test. Data analyses were performed using the SPSS version 9.0 software program for Windows. The significant difference was set as $P<0.05$.

RESULTS

Nitrite Concentrations During Fermentation of Pure Starter Cultures: Changing in nitrite concentration during fermentation period by pure LAB starter cultures is shown in figure 1. All of them were capable to reduce nitrite concentration. *L. plantarum, L. fermentum and Leu. mesentroides* depleted 91.7%, 99.3% and 75% of the initial nitrite respectively. Nitrite concentration of standard MRS broth inoculated with *Leu. mesentroides* was higher than that of inoculated with *L. plantarum* and *L. fermentum* ($P<0.05$), the nitrite concentration was approximately 30 mg/l at the end of fermentation whereas *L. plantarum* reduced nitrite concentration from 120 to 10 mg/l at the end of fermentation. On the contrary, in standard MRS broth inoculated with *L. fermentum* fluctuation showed on first day, nitrite concentration was approximately 8 mg/l at the end of fermentation period. No significant difference ($P>0.05$) was observed between the residual nitrite concentration of standard MRS broth inoculated by *L. plantarum* and *L. fermentum* at the end of fermentation period. However, these results demonstrated that lactobacillus strains of Heterofermentatives are more effective to reduce nitrite concentration inMRS broth, *L. plantarum* is belonged to Estreptobacterium (Facultative Heterofermentative). Nitrite concentration was nearly constant in control tube as is shown in the figure 1.

Nitrite Concentrations During Fermentation of Mixed Starter Cultures: Changes in nitrite concentration during fermentation period by mixed LAB starter cultures are shown in figure 2. All of them were capable to reduce nitrite concentration ($P<0.05$). *L. plantarum* and *L. fermentum*, *L. plantarum* and *Leu. mesentroides*, *L. fermentum* and *Leu. mesentroides* mixtures depleted nitrite concentration 98.3%, 83.3% and 89.2% respectively.

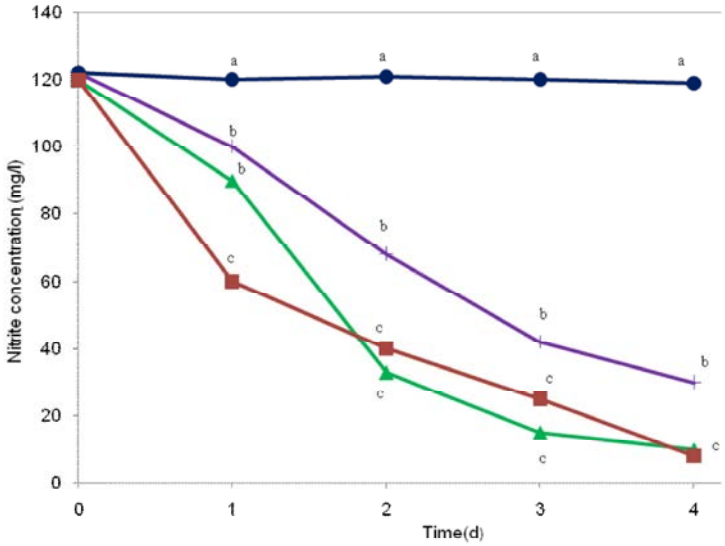

Fig. 1: Nitrite depletion by pure LAB in standard MRS-sodium nitrite solution during fermentation period. Symbol: Control: (?), *L. plantarum*: (|), *L. fermrntum*: (?), *Leu. mesentroides*: (×). a-c: Means in the same time with unlike letters are significant different ($P<0.05$).

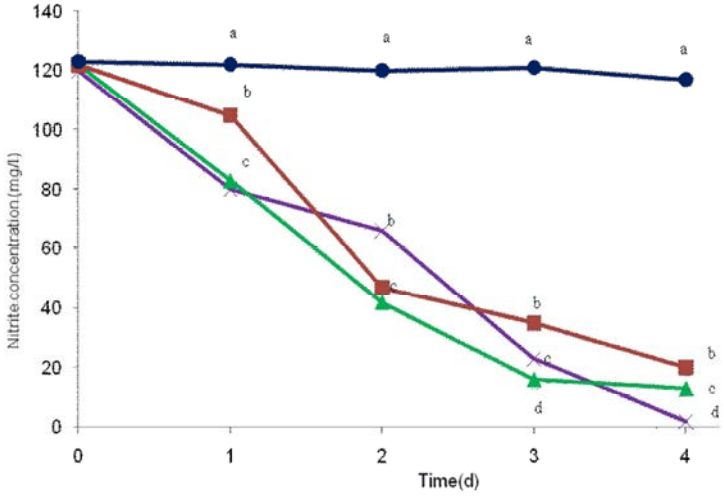

Fig. 2: Nitrite depletion by mixed LAB in standard MRS-sodium nitrite solution during fermentation period. Symbol: Control:: (?), *L. plantarum* and *L. fermrntum*:(×), *L. plantarum* and *Leu. mesentroides*:(|), *L. fermenum* and *Leu. mesentroides*: (?). a-d: Means in the same time with unlike letters are significant different ($P<0.05$).

The results demonstrated that standard MRS broth inoculated with *L. plantarum* and *Leu. mesentroides* mixture have the highest nitrite concentration in spite of decreasing at 48 initial hours of incubation, the nitrite concentration was approximately 20 mg/l at the end of fermentation. *L. plantarum* and *L. fermentum* mixture decreased ($P<0.05$) sharply and reduced nitrite concentration from 120 to 2 mg/l. At the end of fermentation, nitrite concentration in standard MRS broth inoculated by *L. fermentum* and *Leu. mesentroides* was 13 mg/l. Some of the LAB mixtures have high potential to reduce nitrite concentration compare to pure LAB starter cultures. Besides Lactobacillus strains mixture decreased nitrite concentration excellently, they apparently have strong synergistic activity for nitrite reduction.

DISCUSSION

As result showed, LAB strains can proliferated in standard MRS-sodium nitrite solution, *Leu. mesentroides* and mixed *L. planratum* and *Leu. mesentroides* starter cultures may were needed more time for adaptation with standard MRS- sodium nitrite. Navarro *et al.* showed that LAB are able to proliferate in media contained nitrite and nitrate [22]. 5 of 10 strains of lactic acid bacteria identified as reference and 16 of 25 strains of lactic acid bacteria isolated from cured meat were stable in nitrite medium without any proliferation changing and there was no significant difference in growth in hemofermentatine strains whereas heterofermentatives had lower proliferation rate initially [23]. Tow mechanisms namely chemical depletion resulting from acid production during LAB growth and enzymatic reduction lead to depletion of nitrite [8]. Nitrite and nitrate reductases are present in certain strains of *Lactobacillus*, they can reduce nitrite to ammonia [4]. In the present study, MRS broth inoculated with *L. fermentum* and *L.plantarum* and *L. fermentum* starter cultures contained the lowest nitrite concentration. In the other study *L. plantarum* decreased nitrite more than *L. fermentum* slightly in MRS broth [24]. Studies by some researchers found that *L. plantarum* has Nitritereductaes activity in both aerobic and anaerobic conditions whereas *L. sakii* and *L. kurvatus* no produce reductase enzyme. In the other hand, lactic acid production by LAB is as important as nitritereductase enzyme production [25,26]. Some researchers demonstrated that some *Lactobacilli* isolated from cured meat products are capable to reduce nitrite enzymatically, they found that LAB isolated from commercial meat samples depleted 61.4-92.7% of nitrite in Adenosine Three Phosphate (ATP) broth under anaerobic conditions and suggested that the production of the lactic acid and the consequent decrease in pH value were partly responsible for the nitrite losses [27]. The mixed *L. plantarum* and *L. fermentum* starter culture was reduced nitrite concentration quickly. Apparently, lactobacilli strains may have high reductase activity and acid production ability although they have strong synergistic activity to reduce nitrite concentration. The mixed LAB starter cultures- *L. plantarum* and *L. fermentum* exception- showed no expected reduction ability and they reduced nitrite concentration in average of 86.2%. It may be related incubation temperature, source of their isolation and their reduction nitrite ability genetically. In the present study,

temperature of incubations were fixed on strain's optimum growth for pure starter cultures and mixed *L. plantarum* and *L. fermentum* starter culture, so *L. plantarum* and *Leu. mesentroides* and *L. fermentum* and *Leu. mesentroides* (incubated at 30?, *leuconostoc*'s optimum) reduced nitrite concentration lower than 90% among of 4 days, besides results demonstrated that *Leu. mesentroides* and *L. plantarum* have low ability to reduce nitrite compared to another strain in standard MRS broth. Some researchers found that the nitrite reducing ability of *L. plantarum*, *L. fermentum* and *Leu. mesentroides* increased with temperature increasing, besides they decreased nitrite concentration up 90% among of 2 days [28-30]. Mixed LAB isolated from spontaneous fermented Paocai depleted only 31.6% of the initial nitrite in MRS broth whereas pure starter culture of *L. pentosus*, *L. plantarum*, *Leu. mesentroides*, *L. brevis*, *L. fermentum* depleted 99.6%, 99.3%,99.2%, 97% and 98.8% of the initial nitrite, respectively in anaerobic condition, at 37? for 3 days [8].

CONCLUSION

In addition to the pure and mixed LAB strains ability to deplete nitrite in MRS broth. The mixed strains of lactobacilli bacteria are more effective than others, pure starter culture of *L. fermentum* andmixed *L. fermentum* and *L. plantarrum* reduced nitrite concentration excellently. However, amount of the nitrite reduction depend on conditions such as: incubation temperature and time, genetically specifications and source of isolation. These ALB strains can be used in commercial starter culture designation, also further researches will be needed to consideration of LAB strains ability in food medium surely.

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