Kufa journal for Veterinary Medical Sciences Vol.(14) No.(2)

Kufa journal for Veterinary Medical Sciences

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The Abilities of Different Lactic Acid Bacterial Stains to Deplete Nitrite Level in MRS and M17 Broth

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Received date: 22 May. 2023 **Accepted:** 17 Sep. 2023 **page:** (18-23) **Published:**30 Dec. 2023

DOI: https://doi.org/10.36326/kjvs/2023/v14i212139

Abstract

Nitrite is still regarded as essentially undesirable in foods. Therefore, it is necessary to keep its concentration under control for food safety. The current study was conducted to evaluate the ability of two reference lactic acid strains to deplete nitrite. *Lactobacillus acidophilus* ROO52 and *Streptococcus thermophiles* YC-180 were examined in De Man Rogosa and Sharpe (MRS) and M17 broth with standard sodium nitrite at 0.10 mg /mL and 1 mL (10^{-8} cells per mL) for each of the reference strains to determine the ability of these strains to eliminate nitrite during the fermentation period. A control group, MRS and M17 broth and standard nitrite without inoculum were used. The nitrite depletion was determined by a UV-Vis spectrophotometer. After 48 hours of fermentation, there were significant differences between residual nitrite levels of broth inoculated by *Streptococcus thermophiles* YC-180 compared to control and *Lactobacillus acidophilus* ROO52. *Streptococcus thermophiles* YC-180 depleted nitrite levels significantly ($P<0.05$) from the initial concentration (0.10 mg/ml) to 0.02 mg/ml with a percentage (45%) after 48 hours compared to control and *Lactobacillus acidophilus* ROO52, which depleted nitrite levels from the initial concentration of 0.10 mg/ml to 0.04 mg/ml with a percentage (22.5%) after 48 hours. In conclusion, *Streptococcus thermophiles* YC-180 was more effective at nitrite depletion than *Lactobacillus acidophilus* ROO52. The reduction of nitrite occurs due to acid production and the enzymatic action of these bacteria.

INTRODUCTION

In many countries, nitrite is regarded as a chemical agent that can be used in a variety of applications, such as fertilizers and food preservatives, to prevent the growth of gas-producing bacteria like *coliform* bacteria that contaminate food [1]. In some kinds of raw food, the presence of nitrite reduces the growth and multiplication of bacteria such as *Staphylococcus aureus*, *E. coli*, and *Listeria monocytogenes*, while the presence of nitrite has no effect on the growth and survival of other lactic acid bacteria strains [2, 3]. Numerous studies have shown that nitrite may contaminate food when it is created by an organism that converts nitrate to nitrite or when it is added to food as an additive [4]. In comparison to nitrate, the content of nitrite in groundwater or surface water is usually minimal [5]. Furthermore, nitrate is not

dangerous to humans when consumed, but it becomes harmful when converted to nitrite, and nitrite can cause methemoglobinemia by oxidizing the ferrous ion in hemoglobin. After that, hemoglobin becomes un-stable and releases oxygen to the tissue, resulting in a decrease in the oxygen capacity. This will lead to cyanosis (blue skin syndrome) and hypoxia (decreased O_2 in blood and tissue) [6]. However, bacteria have the ability to reduce nitrate to nitrite at the acidity pH in the stomach of adults, and nitrite can react with the acid and amines in the stomach to produce nitrosamine [7]. In addition, nitrate can be reduced to nitrite in the plants during fermentation [8]. Nitrosamines also have also been indicated to be related to gastric, esophagus, colon cancer and other tumors [9]. Several attempts have focused to reduce nitrite concentration in food. Lactic acid bacteria have been discovered to contribute to the nitrite reduction in some foods [8]. Nitrite can be degraded by the action of lactic acid bacteria due to acid and enzyme degradation [10]. The current study deals with the determination of the nitrite level in the MRS and M17 broths and the ability of reference lactic acid bacterial strains to eliminate and/or deplete it.

MATERIALS AND METHODS

Ethical statement:

No interventions were needed in this study, so there is no need for ethical approval.

Preparation of media for the activation of lactic acid bacteria (LAB):

Both MRS agar and broth were used to promote the activation of *Lactobacillus acidophilus* ROO52 obtained from (ATCC). MRS agar was prepared according to the manufacturer's company (Oxiod) by suspending 62 grams of MRS agar in 1 litter of deionized water (DW). It was boiled to completely dissolve by magnetic stir, and then sterilized by autoclave for 15 minutes at 121 ˚C. Cooled at 50 ˚C or less and poured into the sterile petridishes [11]. The M17 medium and broth (selective media for *Streptococcus thermophiles*) (Himedia) were prepared according to the manufacturing company to promote the activation of *Streptococcus thermophiles* YC-180 obtained from (ATCC). About 55 grams of medium suspended in one liter of deionized water. Thoroughly mixed, dissolved by magnetic sterrrie, and sterilized in an autoclave for 15 minutes at 121 ˚C. Then cooled at (40 to 45 ˚C) and finally poured into sterilized plates.

Activation of reference strains of LAB:

Under the aseptic environment, reference strains of LAB were activated in the milk hygiene laboratory/ college of veterinary medicine/ University of Baghdad. Aseptically revived the lyophilized bacteria of the references strain of both *Lactobacillus acidophilus* ROO52 and *Streptococcus thermophiles* YC-180 obtained from (ATCC) by added about 2 grams from each bacteria to the 25 mL of the sterile MRS and M17 broth respectively, mixed by vortex, and incubated in anaerobic conditions at 37 ˚C for 24-48 hours, 1 ml of bacteria added to 9 ml of MRS and M17 broth.

The procedure was repeated three times. Each tube was cultivated with the Spread Plat Method (SPM) on MRS agar for *L. acidophilus* and M17 agar for *S. thermophiles* and incubated for 24-48 hours under suitable anaerobic conditions. *L. acidophilus* appeared as creamy or beige pigment, large colonies with a little sticky consistency and a smooth surface. The tubes were para film-sealed and kept in the refrigerator. [12].

Preparation of reference lactic acid bacterial broth:

McFarland standards were used to estimate the cell concentration in a suspension visually. McFarland (0.5) was prepared by combining 0.5 ml of a 1.175% (w/v) barium chloride dehydrate ($BaCl2.\ 2H_2O$) solution (Himedia) with 99.5 ml of 1% (v/v) sulfuric acid (H_2SO_4) (Himedia). The turbidity of the standard is similar to that used to prepare the inoculum suspension when aliquoted into test tubes [13]. Using the spectrophotometer (Optima) with a 1 cm light path, the accuracy of the density of a McFarland standard was checked; the absorbance of standard McFarland at a wavelength of 600 nm was 0.132.

Preparation of bacterial suspension:

Three colonies from *Lactobacillus acidophilus* ROO52 and *Streptococcus thermophiles* YC-180 were harvested with an inoculating loop on an overnight culture of 18-24 hours and thoroughly transferred the growth to a tube of MRS and M17 broths, respectively, and vortexes well. About 0.5 McFarland standard were compared to the bacterial suspension, this comparison was seen against a white paper sheet with sharp black lines drawn on it. Bacterial suspensions were adjusted and confirmed the accuracy of the McFarland standard, then preparing serial 10-fold dilutions, for conducting the plate counts [14].

The ability of the reference strains of LAB to deplete sodium nitrite:

Bacterial cells' depletion of sodium nitrite in MRS and M17 broths was calculated using the Dodds and Collins-Thompson [15] method. The ability of LAB to deplete sodium nitrite $NaNO_2$ was determined in the cultural broth. Standard sodium nitrite solution was prepared by adding 100 grams of sodium nitrite (purity 99.0%) purchased from Sigma-Aldrich (USA) to a liter of distilled water. One ml of 0.10 mg/mL was added into 9 ml of MRS and M17 broths severally (as standard broth-sodium nitrite solution) with LAB strains were freshly cultured and inoculated with 1 ml of bacterial suspension at concentration of 10^{-8} cfu/ml of both *Lactobacillus acidophilus* ROO52 and *Streptococcus thermophiles* YC-180 were added to the screw-cap tube severally. As a control, standard nitrite without inoculum and MRS and M17 broths were used [8].

Tubes with only MRS and M17 broth were used as a blanks. All tubes were incubated anaerobically for 0, 24 and 48 hours at 37 C. Yan *et al.*, [8] described a method for measuring primary and final absorbance at 530 nm using the spectrophotometric nitrite method. The mixture was placed in a dark position for 5 minutes at 37 ˚C. The color mixture's optical density was measured at 530 nm against a blank. The nitrite assay was determined by spectrophotometer (Optima). Two absorbance values of both blank and sample filtrate solutions were read at 530 nm with replication.

The nitrite depletion was obtained by the following equation:

$$Nitrite\ depletion\% = (1 - \frac{Ci}{Cf}) \times 100$$ [8].

Ci: the level of nitrite in the broth at 0 h.

Cf: the level of nitrite in the broth after 48 h.

Statistical analysis:

Statistical data analysis was performed using SAS (Statistical Analysis System - version 9.1). Two-way analysis of variance (ANOVA) and Least significant differences (LSD) post hoc tests were performed to assess significant differences among means for more than two groups [16].

RESULTS AND DISCUSSION

The changes in nitrite concentration during fermentation by two reference strains of lactic acid bacteria (LAB) *Lactobacillus acidophilus* ROO52 and *Streptococcus thermophiles* YC-180 are shown in Table 1. *Lactobacillus acidophilus* and *Streptococcus thermophiles* were depleted of 22.5% and 45% respectively, of their initial levels of nitrite. After 24 hours of fermentation, standard MRS broth inoculated with *Lactobacillus acidophilus* ROO52 with a known nitrite concentration had a higher reduction in nitrite concentration than M17 broth inoculated with *Streptococcus thermophiles* ($P<0.05$). After 48 hours, there was a significant reduction ($P<0.05$) in the residual nitrite levels of standard M17 broth inoculated by *S. thermophiles* YC-180 compared to control and MRS broth inoculated by *Lactobacillus acidophilus* ROO52. *S. thermophiles* YC-180 in the M17 broth depleted nitrite levels significantly ($P<0.05$) from the initial concentration (0.10 mg/ml) to 0.02 mg/ml with percentage (45%) compared to control and *Lactobacillus acidophilus* ROO52 in the MRS broth, which depleted nitrite levels from the initial concentration (0.10 mg/ml) to 0.04 mg/ml with a percentage (22.5%) after 48h as we shown in Table 1.

Table 1. Effect of references strains of lactic acid bacteria (*Lactobacillus acidophilus* ROO52) and (*Streptococcus thermophiles* YC-180) in the MRS and M17 broth on the nitrite depletion after anaerobic condition.

Bacteria	Contact time			Depletion %
	Zero time Mean ± SE	24 h Mean ± SE	48 h Mean ± SE	
Control	A 0.10 ± 0.007 a	A 0.10 ± 0.004 a	A 0.09 ± 0.004 a	1%
***L. acidophilus* ROO52**	A 0.10± 0.010 a	A 0.08 ± 0.02 a	B 0.04 ± 0.004 b	22.5%
***S. thermophiles* YC-180**	A 0.10 ± 0.004 a	A 0.09± 0.004 ab	B 0.02 ± 0.004 b	45%
LSD	0.15			

Means in the same column with different small letters differ significantly (P0.05)
Means in the same raw with different capital letters differ significantly (P<0.05)

The result of current study showed the ability of reference LAB to reduce the nitrite levels in MRS and M17 broth during fermentation stage. LAB can grow in a nitrite-containing medium; According to Navarro *et aL* [17] nitrite depletion is caused by two mechanisms: chemical depletion as a result of acid production during LAB development and enzymatic reduction [8]. Hongfu *et al.,* [18] found that nitrite levels were first reduced by enzyme during the first stage of fermentation, and then by acid during the second stage of LAB fermentation. Certain *Lactobacillus* strains contain nitrite and nitrate reductase, which can reduce nitrite to ammonia in the process known as fermentative nitrate reduction [14]. LAB contains a reductase enzyme, which reduces nitrite in an anaerobic environment, indicating that LAB contributes to the depletion of nitrite in foods [19]. This is a relevant fact for food safety [20] to reduce the utilization of nitrates and nitrites in food preservation. Some researchers have found that *Lactobacilli* isolated from cured meat products can reduce nitrite enzymatically. They discovered that LAB isolated from commercial meat samples depleted 61.4-92.7 % of nitrite in Adenosine Triphosphate (ATP) broth under anaerobic conditions, suggesting that lactic acid production and the resulting decrease in pH value were partly responsible for nitrite degradation [15]. LAB breaks down carbohydrates into a range of organic acids, including lactic, acetic, and propionic acids, all of which contribute to the pleasant taste of fermented foods [21]. Therefore, to ensure the safety and quality of fermented products, manufacturers use not only a starting culture, but also curing agents like nitrite to develop color, suppress pathogenic bacteria, and delay rancidity [22].

The result demonstrated that LAB had the ability to deplete the level of nitrite, which was in agreement with Dodds and Collins, [15] they compared two different strains of LAB including *L. acidophilus* and *L. lactis* and reported that these LAB can deplete 92.7% and 61.4% respectively, of the initial nitrite concentration (200ppm) after 24 and 30 hours in broth medium under anaerobic conditions, and they attributed this depletion in nitrite to degradation of nitrite by nitrite-eliminating bacteria, particularly LAB. However, Awort *et al*., [23] explain the degradation of nitrite due to the reduction of nitrite to gaseous nitrogen. The lowering of nitrite during the fermentation stage was attributed to the conversion of nitrite to nitric oxide (O), nitrous acid, and nitrates

[24]. According to Fournaud *et al*., [25], the elimination of nitrite could be due to the ability of certain lactobacilli strains to convert nitrite to nitric oxide, nitrous oxide, or nitrogen. The nitrite depletion that occurs in the control group (1%) may be due to lowering the pH during storage, as the reduction of nitrites is increased when the pH is lowered by 0.2 units [26].

CONCLUSION

References stains of lactic acid bacteria that were used in the current study had the ability to deplete the nitrite concentration due to the fermentation process and *Streptococcus thermophiles* YC-180 had more nitrite depletion (45%) than *Lactobacillus acidophilus* ROO-52 depletion (22.5%) on the M17 and MRS broths respectively.

ACKNOWLEDGMENTS

I would like to thank all the people in the Department of public health, College of Veterinary Medicine, University of Baghdad, Iraq, who made this work possible.

CONFLICT OF INTEREST

There were no reported conflicts of interest in this study.

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