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## Effect of inoculation of *Lactobacillus acidophilus* on the level of nitrite and nitrate in yogurt containing spinach during refrigeration in 21 days

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### ABSTRACT

**Introduction:** Yogurt is mixed with spinach in some countries. Spinach can be a probable dietary source of Nitrate and Nitrite, and further N-nitrosamine.

**Methods:** The present study aimed to investigate the effect of inoculation of *Lactobacillus acidophilus* on the level of nitrite and nitrate in “yogurt containing spinach” (YS) during refrigeration in for 21 days. 4 types of yogurts including yogurt (Y), probiotic yogurt (PY), YS, and probiotic YS (PYS) were produced and kept at 4 °C for 21 days. Their nitrite and nitrate levels were measured by HPLC on days 1, 7, 14, and 21.

**Results:** There was no significant difference between the mean concentrations of nitrate and nitrite of Y and PY and between YS and PYS. Although the effect of refrigeration at 4 °C for 21 days was not significant on the nitrate amount of Y and PY, it was close to a significant level in YS and PYS. It also caused a significant increase in the nitrite content of all

yogurts. This study showed that *Lactobacillus acidophilus* bacteria cannot affect the amount of nitrate and nitrite in yogurt or YS at 4°C.

**Conclusion:** Increasing the nitrite content of samples may enhance the possibility of nitrosamine formation. It is suggested that those who are at risk for all malformations caused by nitrites should not keep YS for a long time.

**Keywords:** nitrate; nitrite; *Lactobacillus acidophilus*; probiotic yogurt containing spinach; HPLC



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## INTRODUCTION

The compounds nitrates and nitrites are present naturally in the environment and can be consumed by humans through meats, vegetables and drinking water. Nitrate can convert into nitrite by bacterial activity [1]. Because of their carcinogenic effect [1-2], they may lead to some diseases including methemoglobinemia in infants [3-4] and congenital malformations in children [5], they have been considered as unwanted compounds in the diet. Furthermore, an increase in the nitrate level leads to block iodide uptake of the sodium iodide symporter [6]; also, it can lead to nutrient deficiency and raise the risk of kidney stones [7]. Many studies have indicated that nitrites can react with secondary amines and form carcinogenic compounds named N-nitrosamines in the stomach [1-2, 8-9]. However, some studies have revealed that nitrite accumulation in the

tissues and blood is a biological source of nitric oxide (NO) which can enhance gastroprotection and reduce blood pressure. It possesses good effects on cardiac function and participate in mitochondrial respiration [10-11]. Nitrates are found in large quantities in vegetables, so that 70-90% of dietary nitrate intake comes from vegetables [12-16]. Green leafy vegetables contain higher levels of nitrate and spinach is one of them [14-15, 17].

One of the forms of spinach consumption in Iran is a combination of cooked spinach with yogurt, and it is not clear what effect has the combination of spinach with yogurt on the amount of nitrite and nitrate; furthermore, the prepared mix may be refrigerated for several days. However, it should be noted that nitrate and nitrite are compounds that have an Acceptable Daily Intake (ADI). The ADI for nitrate is 3.7 mg kg<sup>-1</sup> day<sup>-1</sup> [3, 15, 18] and for

nitrite it is 0.07 mg kg<sup>-1</sup> day<sup>-1</sup> [19]. It means that the nitrate intake for a 60 kg adult should not be higher than 222 mg nitrate per day [3, 14-15, 18-19]. Therefore, nitrate and nitrite intake should be minimized, especially for those who consume lots of vegetables in their diet [18].

Today, a part of yogurt production in the world is in the form of probiotics. Probiotics are live microbial food supplements that have benefits for health [20]. Some beneficial effects of probiotic consumption include improvement of digestive system health, reducing the risk of certain cancers, contributing to relief from lactose intolerance symptoms [21-22], enhancement of the immune system, synthesis and enhancement of the bioavailability of nutrients, decreasing prevalence of allergies in susceptible individuals [22], anti-diarrhea effects [21, 23] and reducing serum cholesterol [21, 23-24]. In addition, the consumption of probiotic yogurt can also reduce the inflammatory, infectious, and metabolic effects of pregnancy [25]. Among the probiotic bacteria, lactic acid bacteria have been identified as the most important one [26].

Studies have shown that lactic acid bacteria are involved in the depletion of nitrate in many foods [4]. Recent findings indicate that lactobacillus can use nitrate and nitrite and produce NO in the digestive tract, which plays a role in physiologic events as told [27-28].

Yogurt is mixed with vegetables such as spinach in some countries, and there is not any study on the effect of yogurt starter and probiotic bacteria activity on the amount of nitrite and nitrate in these yogurts during the storage period in the refrigerator. Also, each kilogram of fresh spinach can contain up to 5 grams of nitrate [5, 29]; and still, there are questions about the usefulness or harmfulness of nitrate. Considering the above points, we aimed to investigate the effect of inoculation of

*Lactobacillus acidophilus* on the level of nitrite and nitrate in yogurt containing spinach during refrigeration for 21 days.

## MATERIALS AND METHODS

**Instruments:** According to Keshavarz et al. [14], the samples were analyzed using Waters HPLC (High-Performance Liquid Chromatography) system (made in waters company, US), which was equipped with a C18 Waters Spherisorb® 5 µm ODS2 (250 × 4.6) column, UV Waters 2487 Dual λ Absorbance Detector and Waters 1525 Binary HPLC Pump. For degassing the solutions, YL9100 HPLC System Vacuum Degasser was utilized. PH measurements were determined using a Metrohm 827 pH meter (Switzerland). Sigma Laboratory Centrifuges 3k30 (Germany) was utilized to centrifuge the samples before injection, and Elmasonic S60H ultrasonic system (Germany) was used for the preparation of samples.

**Chemicals:** All reagents were prepared using Merck Company (Germany). The YC-X11 yogurt starter (including *Streptococcus thermophilus* and *Lactobacillus bulgaricus*) and *Lactobacillus acidophilus* LA-5 produced by Hansen Company (Denmark) are used to produce a simple yogurt and probiotic yogurt.

## Preparation of samples

- 1. Preparation of spinach:** According to Keshavarz et al. [14], spinach was bought from green groceries and after removing their visible soil and non-edible parts, they were cut into 4-7 cm pieces. Then, they were washed with deionized water and drained. They were heated in a closed container for about 40 min without adding water.
- 2. Preparation of 4 types of yogurts:** To prepare

yogurt, milk (3.2% fat) was heated at 90 ° C for 10 minutes. The heated milk was then divided into two parts. In one part, for one liter of milk, 0.05 gr of lyophilized powder of *Lactobacillus acidophilus* with 0.05 gr of lyophilized starter powder of yogurt, and in the other part, only starter powder of yogurt (0.1 g per liter of milk) was inoculated as the control. The milk was stored in 250 g disposable containers at 40°C incubator until the pH reached 4.6 and then transferred to the refrigerator. On the next day, some cooked spinach (in the amount of 15% by weight) was added to half of the simple and probiotic obtained yogurts. Then, all the samples including simple yogurt (Y), probiotic yogurt (PY), yogurt containing 15% cooked spinach (YS) and probiotic yogurt containing 15% cooked spinach (PYS) were kept in the 4°C refrigerator for 21 days.

**Sample preparation for analysis:** After the initial preparation, the samples were prepared for HPLC injection based on the National Food Safety Standard of the People's Republic of China [30]. All glassware was base-washed by NaOH solution and then washed with deionized water several times.

**Preparation of mobile phase and standard solutions:**

Various aqueous methanol concentrations (40, 30, 20, 15 and 10% v/v) and different pH values (4.5 - 7.5) were tested. PH adjustment was done by adding orthophosphoric acid. Different amounts of Octylamine (0.005, 0.0075, 0.010, 0.0125, 0.015, 0.020, 0.025 and 0.030 M) were added to the solutions. Finally, the solutions were passed from a 0.22µm filter. Eventually, the optimal condition of the mobile phase (10%

methanol, pH 6.5 with 0.0125 M Octylamine) was used in the experiment. Standard solutions which were prepared on the day of the experiment and injected into the HPLC system contained 3.12, 6.25, 12.5, 25 and 50 µg/mL of nitrite and nitrate.

**HPLC analysis:** After testing different flow rates (0.5, 0.8, 1 ml min<sup>-1</sup>), the optimum flow rate was obtained 0.8 ml min<sup>-1</sup>. The injection volume and the UV wavelength were 5 µL and 213 nm, respectively. HPLC column was washed and refreshed by passing a mixture solution of water and methanol (50:50 v/v) at the end of the analysis.

**Statistical analysis:** The obtained data were analyzed using SPSS, version 18. Descriptive statistics were used to present the mean and standard deviation. ANOVA with repeated measurements was used to compare the changes in nitrate and nitrite of Y, PY, YS and PYS overtime during days 1, 7, 14 and 21. One-way ANOVA test was used to compare the changes in nitrate and nitrite of groups on each measurement day. The significance level was  $P < 0.05$ .

**RESULTS**

After examining various conditions to set up the HPLC system, the optimum mobile phase for detection and separation of two peaks of nitrate and nitrite was 10% (v/v) aqueous methanol with the addition of 0.0125 M Octylamine which was adjusted in 6.5pH value. The flow rate was 0.8 ml min<sup>-1</sup>.

Table 1 shows nitrate changes in Y, PY, YS and PYS during the refrigeration storage period. The changes were not significant in any of the groups over time although in the PYS group it was close to a significant level ( $P=0.058$ ). The trend of nitrate changes over time in the two groups of Y and PY ( $P= 0.50$ ) and also in the two

groups of YS and PYS ( $P=0.986$ ) was similar. The amount of nitrate in the two groups of YS and PYS increased in the first and second weeks and decreased in the third week, and in the two groups of Y and PY this increase was seen

only in the first week, and after that, the amount of nitrate was reduced. However, none of these changes was significant.

**Table 1.** Changes in nitrate level of yogurt, probiotic yogurt, yogurt containing spinach and probiotic yogurt containing spinach during refrigeration storage at 4 °C in 21 days

groups	n	level of NO <sub>3</sub> during refrigeration storage				p value
		1st day	7th day	14th day	21th day	
		Mean ± SD (ppm)	Mean ± SD (ppm)	Mean ± SD (ppm)	Mean ± SD (ppm)	
Y	6	11.74 ± 6.75 <sup>a</sup>	18.46 ± 6.87 <sup>a</sup>	14.36 ± 2.92 <sup>a</sup>	11.91 ± 6.57 <sup>a</sup>	<b>0.092</b>
PY	6	13.32 ± 5.26 <sup>a</sup>	13.60 ± 8.30 <sup>a</sup>	10.93 ± 5.73 <sup>a</sup>	9.63 ± 2.74 <sup>a</sup>	<b>0.609</b>
YS	6	102.18 ± 29.56 <sup>b</sup>	133.55 ± 14.38 <sup>b</sup>	139.14 ± 8.91 <sup>b</sup>	136.01 ± 8.12 <sup>b</sup>	<b>0.071</b>
PYS	6	104.38 ± 17.51 <sup>b</sup>	133.89 ± 9.73 <sup>b</sup>	139.47 ± 28.83 <sup>b</sup>	137.91 ± 9.06 <sup>b</sup>	<b>0.058</b>

Y: yogurt; PY: Probiotic yogurt; YS: yogurt containing spinach; PYS: Probiotic yogurt containing spinach

Differences between values in columns signed with the same letters are non-significant

Table 2 shows nitrite changes in Y, PY, YS and PYS during the refrigeration storage period. The trend of nitrite changes in each of the groups was significant during the storage period in the 4°C refrigerator.

However, there was no significant difference in the trend of nitrite changes during the time between the groups ( $P = 0.252$ ).

**Table 2.** Changes in nitrite level of yogurt, probiotic yogurt, yogurt containing spinach and probiotic yogurt containing spinach during refrigeration storage at 4°C in 21 days

groups	n	level of NO <sub>2</sub> during refrigeration storage				p value
		1st day	7th day	14th day	21th day	
		Mean ± SD (ppm)	Mean ± SD (ppm)	Mean ± SD (ppm)	Mean ± SD (ppm)	
Y	6	39.25 ± 8.64 <sup>a</sup>	50.56 ± 8.80 <sup>a</sup>	56.71 ± 5.85 <sup>b</sup>	45.08 ± 7.53 <sup>ba</sup>	<b>0.004</b>
P.Y	6	38.28 ± 8.03 <sup>a</sup>	46.87 ± 7.00 <sup>a</sup>	48.59 ± 7.15 <sup>ba</sup>	41.01 ± 2.25 <sup>a</sup>	<b>0.040</b>
YS	6	35.44 ± 12.50 <sup>a</sup>	47.35 ± 6.71 <sup>a</sup>	50.75 ± 4.87 <sup>ba</sup>	46.17 ± 5.42 <sup>ba</sup>	<b>0.034</b>
P.YS	6	34.72 ± 10.05 <sup>a</sup>	48.88 ± 2.72 <sup>a</sup>	44.90 ± 4.26 <sup>a</sup>	49.98 ± 3.13 <sup>b</sup>	<b>0.001</b>

Y: yogurt; P.Y: Probiotic yogurt; YS: yogurt containing spinach; P.YS: Probiotic yogurt containing spinach

Differences between values in columns signed with the same letters are non-significant

## DISCUSSION

The aim of this study was to evaluate the effect of

probiotic bacteria, *Lactobacillus acidophilus*, on the amount of nitrate and nitrite in yogurt containing

spinach. The mean nitrate concentration of yogurt on the second day after the production was  $11.74 \pm 6.75 \text{ mg kg}^{-1}$ . It is similar to the results of Kucukcetin et al.'s study, which measured the nitrate of strained yogurt at  $16.6 \text{ mg kg}^{-1}$  [31]. However, it was higher than the nitrate content reported by Martins et al. in yogurt. They reported the nitrate of yogurt was  $3.07\text{-}4.77 \text{ mg kg}^{-1}$  by spectrophotometry and  $0.49\text{-}3.69$  by flow injection analysis (FIA) [32]. Also, Luf et al. determined the nitrite concentration of yogurt, about  $0.72 \text{ mg kg}^{-1}$  [33]. Therefore, the nitrate concentration of yogurt in this study was similar to the results of some studies [31] although was high compared to the other two studies [32, 33]. The mean nitrite concentration of yogurt in this study on the second day was obtained at  $38.28 \pm 8.03 \text{ mg kg}^{-1}$ , which is high in comparison to the nitrite content of yogurt reported in other studies. Kucukcetin et al. reported the mean nitrite concentration of strained yogurt as about  $2.1 \text{ mg kg}^{-1}$  [31] and it was reported  $0.43\text{-}0.93 \text{ mg kg}^{-1}$  and  $1.06\text{-}1.86 \text{ mg kg}^{-1}$  in yogurt by Martins et al. [32] and  $0.002 \text{ mg kg}^{-1}$  by Luf et al. [33]. A higher amount of nitrate in dairy products can be due to the water or gas used in the processing or because of the use of nitric acid as a cleaning agent in the factories [33, 34]. Also, milk contamination with nitrate can occur during or after secretion. Since the nitrate of forage is carried to milk in a low amount, most nitrate contamination has a post-secretory route [33]. As Kammerer et al. showed, contamination of the drinking water of cows with nitrates could not significantly change the nitrate levels of their milk [35]. However, Baranová et al. found that after feeding potassium nitrate ( $\text{KNO}_3$ ) to dairy cows, a considerable increase in nitrate of milk was observed, depending on the amount of  $\text{KNO}_3$  [36]. According to Bouchard's study, increased concentration of nitrate and nitrite in milk can also be due to endotoxin-induced mastitis [37]. Therefore, although the amount of nitrate

and nitrite in milk and dairy products is very low in normal conditions, for other reasons, this can be higher than usual. To find the main reason for the higher nitrate and nitrite levels in the yogurts tested in this study, further evaluation should be done.

In this study, the mean nitrate and nitrite levels between samples of Y and PY groups were not significantly different on any days of measurement, as were not between samples of YS and PYS groups. This indicates that the *Lactobacillus acidophilus* bacteria could not affect the amount of nitrate and nitrite in yogurt or yogurt containing spinach at  $4^\circ\text{C}$ . The effect of *Lactobacillus acidophilus* on the nitrate and nitrite content of Y and YS has not been studied yet, but there are some studies that investigated the effect of other lactic acid bacteria on nitrate and nitrite content of other foods; the results are different depending on the type of bacteria and foods. Gierschner and Hammes showed that the two bacteria, *Lactobacillus plantarum* and *Lactobacillus pentosus*, could not reduce the amount of nitrate in the vegetable extract. Andersson also showed that inoculation of *Lactobacillus plantarum* had no effect on the nitrate content of carrot, but the gram-negative flora of carrot reduced it [38]. However, Hybenova et al. observed that 92H and 90H strains of *Lactobacillus plantarum* and 37 H strain of *Lactobacillus delbrueckii* bacteria effectively reduced the nitrate level in carrot and cabbage extract; the highest nitrate reduction occurred by strain 92H of *Lactobacillus plantarum* [39].

In another study, Yan et al. examined the effect of inoculating lactic acid bacteria starter cultures on the concentration of nitrite in fermenting Chinese Paocai and observed that in addition to their ability to deplete nitrite, by preventing the growth of nitrate-reducing bacteria such as Enterobacteria, lactic acid bacteria can reduce the formation of nitrite during fermentation of paocai (at  $30^\circ\text{C}$  for 10 days) [4]. Oh et al. investigated the

depletion of sodium nitrite in Lactobacilli MRS broth by lactic acid bacteria isolated from kimchi at different temperatures; they found that these bacteria are very effective in depleting sodium nitrite at high temperatures, so that more than 90% of sodium nitrite was depleted in the initial inoculation period (1-2 days) at 30 °C and 36 °C. However, as the temperature dropped, sodium nitrite depletion was reduced by all species so that at 5 °C, bacteria deplete less than 20% of sodium nitrite after 10 days [40]. In addition to the type of food and the type of bacteria inoculated, the difference in the results of this study and some other studies can be explained by the storage conditions of food, including temperature and storage time. The storage temperature of all types of yogurts in this study was 4 °C, which reduces the ability of bacteria to deplete nitrite according to Oh et al. [40]. Another probable cause is the reduction of the survival of probiotics in yogurt [41] affected by factors such as pH reduction and organic acid accumulation during growth in the storage period [42]; there are some studies that show that the pH of samples of probiotic yogurt decreased during storage at 4°C, and their acidity increased [43-45]. Another factor can be the inhibitory effect of nitrate and nitrite on bacterial growth; it has been shown in a study that nitrite can inhibit the growth of bacteria in certain concentrations and pH [46].

Although the effect of refrigeration at 4 °C for 21 days was not significant on the nitrate amount of Y and PY, it was close to a significant level in YS and PYS ( $p = 0.071$  and  $p = 0.058$  respectively). It caused a significant increase in nitrite content of all yogurts up to the third week ( $p < 0.05$ ). The probable cause of increased nitrate (although wasn't significant) and nitrite during the refrigeration storage period can be evaporation of water and, consequently, the concentration of nitrate and nitrite in the samples. Spinach tissue decomposition by

starter and probiotic bacterial enzymes and therefore the release of nitrate and nitrite from its tissue can also be one of the reasons for the increase of these two compounds. Anyway, this issue may increase the possibility of nitrosamine formation in yogurt, due to the high amount of protein and some nitrosatable amines in yogurt [47].

The mean nitrate concentrations of two groups of Y and YS, and two groups of PY and PYS were significantly different on all measuring days; this suggests that the addition of spinach influences the nitrite levels of yogurt, which seems to be natural due to the high amount of nitrate in spinach [14]. However, the mean nitrite concentrations were almost the same in all groups and did not have a significant difference. Since the amount of spinach added to yogurts in this study was 15 percent, and according to the amount of nitrite in the cooked spinach [14], this result is explainable.

## CONCLUSION

This study showed that *Lactobacillus acidophilus* bacteria cannot affect the amount of nitrate and nitrite in yogurt or yogurt containing spinach at temperatures of 4°C. However, considering the effects of other probiotic bacteria on nitrate and nitrite in other studies, it is possible that other probiotic bacteria can affect the amount of nitrate and nitrite of Y and YS.

Although refrigeration at 4 °C for 21 days does not significantly affect the amount of nitrate, it causes an increment in the level of nitrite in samples up to the third week, and this issue may increase the possibility of nitrosamine formation. Therefore, it is advisable that those who are at risk for cancer and other malformations caused by nitrites should not keep yogurt for a long time.

**List of Abbreviations:** Yogurt: Y; Yogurt containing spinach: YS; probiotic yogurt: PY; probiotic yogurt



containing spinach: PYS; nitric oxide: NO; acceptable daily intake: ADI; High Performance Liquid Chromatography: HPLC; potassium nitrate: KNO<sub>3</sub>

**Competing Interests:** The authors declare that there is no conflict of interest.

**Author Contributions:** SMM: conceptualization, methodology and funding acquisition. MK, RBB & MHY: validation and writing-review and editing. MK: formal analysis, investigation, data curation and writing-original draft preparation.

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