Effects of Natamycin and Temperature on the Microbial Population of Doogh during the Shelf Life

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ARTICLE INFO	ABSTRACT
	Introduction: Due to the development of dairy industries, the consumption of Doogh has risen in Iran as a functional fermented beverage. Use of preservatives to reduce microbial contamination in Doogh could increase the risk of chronic diseases in the long run. The present study aimed to extensively investigate the effects of natamycin as a preservative on the microbial population of Iranian Doogh at various storage temperatures. **Methods:** Microbial analyses were performed to determine the total count of microorganisms, population of fungi (molds and yeasts), coliforms, *Escherichia coli*, coagulase-positive staphylococci, and lactic acid bacteria in Doogh samples. **Results:** The microbial population in the Doogh samples (with and without natamycin treatment) stored at 4°C was compared to the samples stored at 25°C and 35°C during the shelf life period. At the temperature of 25°C, natamycin could significantly control the growth of a wide range of microorganisms ($P<0.05$), while at 35°C, in the presence of natamycin, the microbial contaminants in the majority of cases were significantly increased ($P<0.05$). **Conclusion:** According to the findings, it can be concluded that paying attention to the microbial quality of raw milk, heat treatment, and cooling systems during transportation, environmental hygienic conditions and storage of the final products at refrigerated temperatures in order to effectively diminish the microbial population of Doogh and preventing spoilage is necessary.

Introduction

Doogh is a fermented milk drink with high consumption in the Iranian community owing to its organoleptic properties, numerous health benefits, and supplying a significant part of the daily requirements of functional components, such as calcium and vitamins B2, B6, and B12. However, inappropriate thermal processing or secondary contamination may lead to the spoilage of Doogh drink by acid-resistant microorganisms, especially yeasts, molds, and bacteria during storage [1-3].

Natural or chemical preservatives are added to food products in order to stop or delay the unwanted changes caused by microbial, enzyme, and chemical factors [4]. Natamycin, which is also known as pimaricin, has antimicrobial effects against a wide range of molds, yeasts, and bacteria and is widely used in the food industry in order to stabilize the quality of the final products [5, 6]. Natamycin has been approved as a natural preservative for various dairy products (e.g., cottage cheese, sour cream, and yogurt) in more than sixty countries [7, 8]. However, the Iranian Food and Drug Administration and Iranian National Standards Organization have banned the use of preservatives in Doogh [9].

Despite the ban on use of preservatives in yoghurt drink, various preservatives are utilized illegally by some producers to eliminate microbial contamination, especially in summer.

Esfandiari et al. (2013) used reverse-phase high-performance liquid chromatography to determine the amount of several preservative (sodium benzoate, potassium sorbate, and natamycin) in the pasteurized Doogh in Iran. Among 39 studied samples, sodium benzoate was observed in all the samples, while natamycin was detected in 10-25% of the samples, and potassium sorbate was detected in none of the samples [10].

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Dzigbordi et al. (2013) examined the effects of various concentrations of natamycin (5, 6, 7, 8, 9, and 10 ppm) on the microbial population of vanilla yogurt during 28 days of storage at 4°C, and the obtained results indicated that the addition of 8 ppm of natamycin could significantly reduce the number of yeasts in vanilla yogurt [11].

In another study, Sarah et al. (2014) investigated the effects of natamycin (10 ppm) on the starter cultures population and number of molds and yeast in yogurt during the shelf life. The obtained results demonstrated that the mean count of *Lactobacillus bulgaricus* increased on day seven of refrigerated storage, while it decreased significantly at the end of the shelf life. In addition, the mean count of *Streptococcus thermophilus* decreased during storage, and no molds and yeasts were isolated after 21 days of storage [12].

The temperature and shelf life of dairy products are potentially correlated, and also these parameters depend on other factors, such as the microbial population and chemical quality of raw milk, additives, pre-treatment of milk, pH of the products, and storage conditions. Thermal treatment is often carried out at the temperature of 60-70°C for a short period, which may not eliminate all the microorganisms from the final product. Furthermore, Doogh may be maintained in food stores at an ambient temperature, which is likely to shorten the shelf life [13, 14].

Some of the most important influential factors in the spoilage of dairy products at factories are lack of cooling equipment, improper manufacturing practices, and poor storage conditions. Unfortunately, the manufacturers of these products tend to use preservatives instead of solving these issues, while the use of these compounds is considered to be major threat to human health in the long run [15].

To date, few studies have investigated the effect of preservatives on the microbial load of dairy products in Iran. The present study aimed to evaluate the interactive effects of natamycin and various storage temperatures (4°C, 25°C, and 35°C) on the microbial population of Doogh during storage.

Materials and Methods

Experimental Materials
The microbial culture media used in the study were supplied by Merck (Germany), including the plate count agar (PCA), yeast extract glucose chloramphenicol (YGC) agar, violet red bile (VRB) agar, lauryl sulfate broth (LSB), eosin methylene blue (EMB) agar, Baird-Parker agar (BPA), De Man, Rogosa, and Sharpe (MRS) agar, anaerobic gas-pack A, and ringer tablets. In addition, eight-centimeter disposable plates were purchased from Labtron (UK).

Sample Preparation
Two types of Doogh samples were prepared at a dairy production plant in Mashhad (Iran), including the samples without natamycin and those containing 10 ppm of natamycin. Both samples were divided into three groups with three different storage temperatures of 4°C, 25°C, and 35°C. The samples in each group were preserved for 60 days at 10-day intervals. All the experimental analyses (microbial counts) were performed in triplicate.

Microbiological Analysis
The microbiological tests that were conducted on the Doogh samples included the total count of microorganisms (National Iranian Standard No. 5272-1) using the PCA, mold and yeast counts (National Iranian Standard No. 10899-1) using the YGC agar, coliform count (National Iranian Standard No. 9263) using the VRB agar, *Escherichia coli* count (National Iranian Standard No. 2946) using the LSB for enrichment and EMB agar as the selective media, coagulase-positive staphylococci count (National Iranian Standard No. 6806-1) using the BPA, and lactic acid bacterial count (National Iranian Standard No. 4721) using the MRS agar under anaerobic conditions at 10-day intervals. All the trials were performed in triplicate [16-21]. For bacterial detection and counting, appropriate serial dilution and culturing were initially performed, and the PCA, EMB, VRB, and BPA media were incubated at the temperature of 32.5°C. In addition, the MRS and YGC media were incubated at the temperature of 37°C and 25°C, respectively for specified periods.

Statistical Analysis
The main variables in the present study were natamycin/non-natamycin treatment and storage temperatures. Each treatment was carried out in triplicate during storage. Data analysis was performed in SPSS version 16 using repeated measures two-way analysis of variance (ANOVA). Moreover, the comparison of means was performed using Duncan's multiple range

test at 5% probability level, and charts were drawn using the Excel software. The obtained values were expressed as mean and standard deviation (SD).

Results and Discussion

Total Count of Microorganisms

According to the information in Table 1, the total count of the samples without natamycin at the temperature of 4°C was lower than 0.5 logarithmic cycle after 10 days, and no count was detected until the end of shelf life. However, in the sample containing natamycin only on day 20, the total count exceeded 10 CFU/ml. According to the obtained results, the total count of the microorganisms in both of the samples preserved at 4°C followed a similar trend.

Table 1. Total count (log cfu/ml)*of pasteurized Doogh samples during storage time

Main Factors		Storage time (day)						
Type of Doogh	Temperature (°C)	1st	10th	20th	30th	40th	50th	60th
Without natamycin	4	0.74±0.04^{Cb}	0.39±0.08^{Bc}	0.00±0.00^{Da}	0.00±0.00^{Aa}	0.00±0.00^{Aa}	0.00±0.00^{Aa}	0.00±0.00^{Aa}
	25	0.74±0.03^{Bb}	1.48±0.08^{Cd}	1.65±0.05^{Dd}	3.70±0.02^{Eb}	3.90±0.04^{Fc}	5.90±0.05^{Gc}	0.00±0.00^{Aa}
	35	0.74±0.04^{Bb}	4.40±0.17^{Ee}	1.00±0.00^{Cb}	0.00±0.00^{Aa}	0.00±0.00^{Aa}	1.20±0.02^{Db}	1.43±0.09^{Ab}
Containing natamycin	4	0.00±0.00^{Aa}	0.00±0.00^{Aa}	1.50±0.07^{Bc}	0.00±0.00^{Aa}	0.00±0.00^{Aa}	0.00±0.00^{Aa}	0.00±0.00^{Aa}
	25	0.00±0.00^{Aa}	0.45±0.05^{Bc}	3.35±0.03^{Df}	0.00±0.00^{Aa}	0.00±0.00^{Aa}	1.20±0.07^{Cb}	0.00±0.00^{Aa}
	35	0.00±0.00^{Aa}	0.23±0.06^{Bb}	2.50±0.02^{De}	3.73±0.03^{Gb}	2.60±0.04^{Eb}	1.27±0.05^{Cb}	2.70±0.00^{Fc}

*Mean ± standard deviation
Different capital letters in each row indicate a significant difference (p<0.05) of interaction between two main factors during shelf life
Different small letters in each column indicate a significant difference (p<0.05) of interaction between two main factors at each storage time

Adebayo et al. (2014) emphasized on the ability of low temperatures on inhibition of the mesophilic microorganisms causing spoilage. They argued that low temperatures (4°C) could prevent the metabolic activity of these microorganisms, thereby reducing their growth or even leading to their death [22].

According to the findings of the current research, the total count of the samples containing natamycin was significantly lower at the temperature of 25°C compared to the natamycin-free samples during all the storage periods (P<0.05). The total count of the samples without natamycin increased until day 50, reaching six logarithmic cycles.

According to the literature, the stability and functionality of natamycin are influenced by various factors, such as pH, temperature, light, and oxidants [23]. Gourama & and Bullerman (1988) reported that natamycin had the most significant inhibitory effects at the temperature of 25-35°C [24]. In the present study, the total count in the natamycin-free samples stored at the temperature of 35°C was high until day 10 of storage, while the most of microorganisms that are involved in food contamination are mesophiles. However, after 10 days of storage at the temperature of 35°C, the presence of natamycin as the preservative not only did not inhibit the activity of the microorganisms in the Doogh samples, it was also recorded to be higher compared to the natamycin-free samples. This phenomenon could be attributed to the production of most antimicrobial compounds from lactic acid bacteria and their effects on other microorganisms, while in the samples containing natamycin, the lactic acid bacteria were suppressed and unable to compete with other microorganisms, thereby increasing the total count significantly [22].

According to the information in Table 1, the total microbial counts were zero in all the treatments during the last storage period, with the exception of the samples that were stored at the temperature of 35°C. This finding could be attributed to the significant reduction of pH due to the activity of various microorganisms during storage. Therefore, lack of nutrients and formation of a strong acidic environment (pH<4) prevented the growth of microorganisms and even led to their disappearance [25]. It is also notable that the Iranian National Standard Organization has not been defined the standard total count for pasteurized Doogh.

Mold and Yeast Population

Fungi are known as major cause of spoilage in yoghurt and other fermented dairy drinks. Due to their low pH, these products also provide a selective condition for the growth of yeasts and molds [11]. According to the information in Table 2, the amount of molds and yeasts in the Doogh samples at the temperature of 4°C was significantly lower in the samples containing natamycin compared to the non-preserved

sample (P<0.05). However, the number of these microorganisms was lower in the natamycin-free Doogh samples until day 40 of storage compared to the standard limit (100 CFU/ml) [17]. On day 50, the count of molds and yeasts increased in the samples without natamycin, which could be due to the consumption of lactate by the fungi and the subsequent increasing pH of Doogh [26].

Table 2. Count of mold & yeast (log cfu/ml)* of pasteurized Doogh samples during storage time

Main Factors		Storage time (day)						
Type of Doogh	Temperature (°C)	1st	10th	20th	30th	40th	50th	60th
Without natamycin	4	0.00±0.00Aa	0.60±0.03Cb	0.60±0.04Cc	0.45±0.09Bb	0.00±0.00Aa	2.94±0.02Db	0.60±0.01Cc
	25	0.00±0.00Aa	1.80±0.08Cc	1.35±0.04Bd	3.80±0.04Dd	3.94±0.02Ec	5.40±0.05Fe	0.00±0.00Aa
	35	0.00±0.00Aa	2.36±0.02Cd	1.33±0.03Bd	0.00±0.00Aa	0.00±0.00Aa	3.54±0.03Dd	0.00±0.00Aa
Containing natamycin	4	0.00±0.00Aa	0.40±0.08 Ba	0.00±0.00Aa	0.39±0.08Bb	0.00±0.00Aa	0.60±0.02Ca	0.00±0.00Aa
	25	0.00±0.00Aa	0.39±0.08Ba	3.35±0.04De	0.00±0.00Aa	0.00±0.00Aa	0.65±0.04Ca	0.47±0.04Bb
	35	0.00±0.00Aa	0.47±0.02Ca	0.23±0.06Bb	3.00±0.01Ec	2.73±0.04Db	3.20±0.02Fc	2.80±0.03Dd

*Mean ± standard deviation
Different capital letters in each row indicate a significant difference (p<0.05) of interaction between two main factors during shelf life
Different small letters in each column indicate a significant difference (p<0.05) of interaction between two main factors at each storage time

As shown in Table 2, the growth of molds and yeasts increased at the temperature of 25°C in the non-preserved Doogh samples, exceeding the maximum of the standard limit after day 20 of storage. In the samples containing natamycin, a significant reduction was observed in the growth of these microorganisms, with the exception of day 20 of storage (P<0.05).

Yeganehzad et al. (2007), Akpan et al. (2007), and Viljoen et al. (2003) have reported that various storage conditions in terms of temperature, microorganism diversity, and preservatives could decrease the pH of the final products [27-29]. Correspondingly, samples containing preservatives possess higher pH-lowering effects, providing the proper condition for the further growth of acid-resistant microorganisms, including molds and yeasts [11, 30].

According to the information in Table 2, no molds and yeasts were observed in the samples without natamycin on day 60 of storage, while in the samples containing natamycin, less than one logarithmic cycle of molds and yeasts was recorded. The number of these microorganisms in the natamycin-free samples could be due to the production of growth inhibitor compounds (e.g., organic acids and hydrogen peroxide) by the other microorganisms found in Doogh [31].

In the current research, natamycin positively influenced the total population of molds and yeasts at the temperature of 35°C until day 20 of storage, while after the mentioned period until the end of storage, natamycin could not decrease fungal contamination; subsequently, the population of molds and yeasts significantly increased. Furthermore, the count of molds and

yeasts in both types of Doogh samples increased significantly on day 50 of production (P<0.05), which could be due to the sporulation activity of these microorganisms at the outset of the first storage period and their conversion into vegetative cells in the middle of the shelf life. Branen et al. (2001) indicated that at the temperature of at 35°C, molds remained as spores on medium containing natamycin on the initial days of storage [23].

According to the results of the present study, the count of molds and yeasts in both types of the Doogh samples stored at the temperature of 35°C significantly decreased at the end of the shelf life (P<0.05), which could be attributed to the reduction of nutrients and unfavorable environmental conditions [31].

Total Coliform Count
Contamination with coliforms after the pasteurization process leads to the growth of these microorganisms at high levels. According to the literature, the presence of coliforms in foods (e.g., dairy products) even in small amounts could cause contamination, ultimately leading to gastrointestinal diseases [32]. In addition, these microorganisms could remain vigorously active during prolonged storage at the temperature of 4°C [33].

According to the information in Table 3, the total count of coliforms was higher than the standard limit (10 CFU/ml) in the non-preserved Doogh samples on day 10 of storage, while in the samples stored at 4°C, the total coliform count was acceptable by the Iranian standards [18]. Since day 30 onwards, the activity of coliforms in both types of Doogh samples could be attributed to the pH decline due to the use of nutrients in the

environment by other microorganisms, as well as the presence of preservative. In a similar study, Jay et al. (2008) reported that the coliform count in the dairy products (e.g., yogurt, butter milk, and sour cream) containing preservatives declined rapidly within three days of storage at the temperature of 7°C, thereby leading to the drastic reduction of pH [25].

According to the results of the present study, the non-preserved Doogh samples had significantly higher levels of coliforms at the storage temperature of 25°C compared to the samples containing the preservative (P<0.05). In the Doogh samples containing natamycin, the coliform count did not exceed the standard limits.

Table 3. Count of coliforms (log cfu/ml)* of pasteurized Doogh samples during storage time

Main Factors		Storage time (day)						
Type of Doogh	Temperature (°C)	1st	10th	20th	30th	40th	50th	60th
Without natamycin	4	0.00±0.00Aa	1.33±0.05Dc	0.92±0.02Cb	0.00±0.00Aa	0.00±0.00Aa	0.00±0.00Aa	060±0.11Bc
	25	0.00±0.00Aa	1.60±0.01Bd	0.00±0.00Aa	3.90±0.02Dd	3.60±0.05Cc	0.00±0.00Aa	0.00±0.00Aa
	35	0.00±0.00Aa	1.96±0.02Ce	0.00±0.00Aa	1.06±0.01Bb	0.00±0.00Aa	0.00±0.00Aa	0.00±0.00Aa
Containing natamycin	4	0.00±0.00Aa	0.90±0.05Ba	0.97±0.02Bc	0.00±0.00Aa	0.00±0.00Aa	0.00±0.00Aa	0.00±0.00Aa
	25	0.00±0.00Aa	1.00±0.03Cb	0.00±0.00Aa	0.00±0.00Aa	0.00±0.00Aa	0.00±0.00Aa	0.40±0.00Bb
	35	0.00±0.00Aa	0.90±0.05Ba	0.00±0.00Aa	2.69±0.08Cc	2.70±0.03Cb	0.00±0.00Aa	2.80±0.01Dd

*Mean ± standard deviation
Different capital letters in each row indicate a significant difference (p<0.05) of interaction between two main factors during shelf life
Different small letters in each column indicate a significant difference (p<0.05) of interaction between two main factors at each storage time

At temperature of 35°C (Table 3), the population of coliforms in the samples without natamycin was less; this could be attributed to the high acid production by the starter cultures due to the lack of natamycin. Increased pH could promote the potential growth of coliform bacteria during production processes [34]. Compared to the samples stored at the temperature of 4°C and 25°C, the coliform count was above the standard limit, especially in the samples containing natamycin.

Our findings demonstrated that the coliform count differed in the samples of Doogh (with and without natamycin), which were preserved at the temperature of 35°C at various shelf life intervals. In general, the lack of uniformity in the growth of these microorganisms could be due to the decrease or increase of pH during growth that are caused by sugar consumption (lactose), production of other inhibitors (e.g., organic acids and hydrogen peroxide), or the consumption of organic acids by other microorganisms [30, 34].

E. coli Count

Escherichia coli is a pathogenic microorganism, which belongs to the Enterobacteriaceae family. Fortunately this microorganism is easily destroyed at pasteurization temperatures, post-contamination with this bacterium after the pasteurization process in dairy products is a substantial challenge to producers [34].

Table 4. Count of *E. coli* (log cfu/ml)* of pasteurized Doogh samples during storage time

Main Factors		Storage time (day)						
Type of Doogh	Temperature (°C)	1st	10th	20th	30th	40th	50th	60th
Without natamycin	4	0.00±0.00Aa	0.40±0.08Bb	0.00±0.00Aa	0.00±0.00Aa	0.00±0.00Aa	0.00±0.00Aa	0.00±0.00Aa
	25	0.00±0.00Aa	1.06±0.01Bd	0.00±0.00Aa	0.00±0.00Aa	0.00±0.00Aa	9.30±0.15Cb	0.00±0.00Aa
	35	0.00±0.00Aa	0.54±0.06Bbc	0.00±0.00Aa	0.00±0.00Aa	0.00±0.00Aa	0.00±0.00Aa	0.00±0.00Aa
Containing natamycin	4	0.00±0.00Aa	0.00±0.00Aa	0.00±0.00Aa	0.00±0.00Aa	0.00±0.00Aa	0.00±0.00Aa	0.00±0.00Aa
	25	0.00±0.00Aa	0.60±0.02Bc	0.00±0.00Aa	0.00±0.00Aa	0.00±0.00Aa	0.00±0.00Aa	0.00±0.00Aa
	35	0.00±0.00Aa	0.60±0.03Bc	0.00±0.00Aa	0.00±0.00Aa	0.00±0.00Aa	0.00±0.00Aa	0.00±0.00Aa

*Mean ± standard deviation
Different capital letters in each row indicate a significant difference (p<0.05) of interaction between two main factors during shelf life
Different small letters in each column indicate a significant difference (p<0.05) of interaction between two main factors at each storage time

As shown in Table 4, *E. coli* was observed in the absence of natamycin at all three storage temperatures (4°C, 25°C, and 35°C) on day 10. At the storage temperature of 25°C, *E. coli* count was higher than one logarithmic cycle, while in the samples containing natamycin maintained at the temperature of 25°C and 35°C, *E. coli* contamination on day 10 was less than one

logarithmic cycle. On day 50, severe *E. coli* contamination was only observed in the natamycin-free samples that were preserved at the temperature of 25°C.

Bachrouri et al. (2002) reported that the rate of *E. coli* in yogurt inoculated with this microorganism reduced to below the detection range after 13 days of storage at the temperature of 4°C, which could be attributed to the presence of acid stress conditions in the fermented dairy products [35].

According to the national standards of Iran, pasteurized Doogh must not be contaminated with *E. coli*. Based on the findings of the current research, it could be stated that only the Doogh sample containing natamycin and preserved at 4°C were standard [19].

Count of Coagulase-positive Staphylococci

According to the Iranian National Standard Organization, the presence and bacterial count of coagulase-positive staphylococci must be evaluated in yoghurt drink [20]. Our findings indicated no contamination with coagulase-positive staphylococci in the samples stored at the temperatures of 4°C, 25°C, and 35°C for two months.

Lactic Acid Bacteria Count

Lactic acid bacteria play a key role in the dairy industry as common parts of many fermented products and starter cultures (McDougall, 2009). Several studies have indicated that these bacteria produce various types of preservative compounds, such as antibiotics, organic acids (lactic acid, stearic acid, and propionic acid), and hydrogen peroxide during the fermentation process. The production of these compounds leads to unfavorable environmental conditions for the growth of the other microorganisms that cause spoilage [36]. After the formulation and pasteurization process, it is expected that lactic acid bacteria will not be detected in the final yoghurt drink product or there will be insignificant levels of these microorganisms detectable.

According to the information in Table 5, lactic acid bacteria count was less than one logarithmic cycle in the Doogh samples with and without natamycin at the temperature of 4°C on days 10 and 20, respectively. After this period, the count of these microorganisms decreased to zero in both types of the Doogh.

At the temperature of 25°C, the lactic acid bacteria count in the samples without natamycin was higher than the samples containing the preservative. Moreover, the highest count of these microorganisms was obtained on day 50 of storage in the natamycin-free Doogh, which was estimated at five logarithmic cycles. After 60 days of storage, no lactic acid bacteria were detected in the samples due to the effect of the preservative compound, pH, and unfavorable environmental conditions [22].

At the temperature of 35°C, the samples with natamycin had significantly higher lactic acid bacterial population compared to the natamycin-free samples ($p < 0.05$).

Table 5. Count of Lactic acid bacteria (log cfu/ml)* of pasteurized Doogh samples during storage time

Main Factors		Storage time (day)						
Type of Doogh	Temperature (°C)	1st	10th	20th	30th	40th	50th	60th
Without natamycin	4	0.00±0.00[Aa]	0.60±0.11[Bc]	0.00±0.00[Aa]	0.00±0.00[Aa]	0.00±0.00[Aa]	0.00±0.00[Aa]	0.00±0.00[Aa]
	25	0.00±0.00[Aa]	1.54±0.04[Bd]	0.00±0.00[Aa]	3.50±0.03[Db]	3.40±0.02[Cc]	5.60±0.03[Ed]	0.00±0.00[Aa]
	35	0.00±0.00[Aa]	2.20±0.04[Ce]	0.46±0.05[Bb]	0.00±0.00[Aa]	0.00±0.00[Aa]	3.14±0.04[Db]	0.00±0.00[Aa]
Containing natamycin	4	0.00±0.00[Aa]	0.40±0.08[Bb]	0.77±0.07[Cc]	0.00±0.00[Aa]	0.00±0.00[Aa]	0.00±0.00[Aa]	0.00±0.00[Aa]
	25	0.00±0.00[Aa]	0.23±0.06[Ba]	3.40±0.02[De]	0.00±0.00[Aa]	0.00±0.00[Aa]	3.14±0.02[Cb]	0.00±0.00[Aa]
	35	0.00±0.00[Aa]	0.60±0.15[Bc]	2.20±0.01[Cd]	3.50±0.02[Fb]	2.88±0.02[Eb]	3.84±0.00[Gc]	2.40±0.05[Db]

*Mean ± standard deviation
Different capital letters in each row indicate a significant difference (p<0.05) of interaction between two main factors during shelf life
Different small letters in each column indicate a significant difference (p<0.05) of interaction between two main factors at each storage time

Conclusion

According to the results, addition of 10 ppm of natamycin to the Doogh samples stored at the temperature of 25°C could control the microbial population, while in the samples stored at the temperature of 35°C, the results were reverse, and the number of the microorganisms increased significantly ($p < 0.05$). In addition, use of a cold chain during the shelf life of the product had a positive impact and could guarantee the microbial quality of the Doogh. Therefore, it is strongly recommended that dairy factories use raw milk with the lowest microbial contamination. On the other hand, adequate heat

treatment, proper cooling equipment (especially in warm seasons), and the establishment of sanitary conditions are the fundamental criteria for the GMP in production lines. Since Doogh is often stored at an ambient temperature and temperature is a key factor in Doogh spoilage, the safest approach to the elimination of preservatives and spoilage symptoms is to preserve these products at refrigerator temperatures in order to effectively improve the quality of the final product.

References

1. Ashraf R, Shah NP. Selective and differential enumerations of Lactobacillus delbrueckii subsp. bulgaricus, Streptococcus thermophilus, Lactobacillus acidophilus, Lactobacillus casei and Bifidobacterium spp. in yoghurt—A review. Int J Food Microbiol. 2011; 149(3): 194-208.
2. Kiani H, Mousavi SMA, Emam-Djomeh Z. Rheological properties of Iranian yoghurt drink, Doogh. International Journal of Dairy Science. 2008; 3(2): 71-8.
3. Athary M, Saeidiasl MR, Elhamirad AH, Armin M. Production of non-carbonated yogurt drink (Dough) enriched with marjoram extract and evaluation of its physicochemical and microbial during storage. Advances in Environmental Biology. 2015; 9(3): 328-34.
4. Saad B, Bari MF, Saleh MI, Ahmad K, Talib MK. Simultaneous determination of preservatives (benzoic acid, sorbic acid, methylparaben and propylparaben) in foodstuffs using high-performance liquid chromatography. J Chromatogr A. 2005; 1073(1-2): 393-7.
5. Reps A, Jedrychowski L, Tomasik J, Winiewska K. Natamycin in ripening cheeses. Pak J Nutr. 2002; 1(5): 243-7.
6. Te Welscher YM, ten Napel HH, Balagué MM, Souza CM, Riezman H, de Kruijff B, et al. Natamycin blocks fungal growth by binding specifically to ergosterol without permeabilizing the membrane. J Biol Chem. 2008; 283(10): 6393-401.
7. Delves-Broughton J. In: Richardson P, editor,Use of the natural food preservatives, nisin and natamycin, to reduce detrimental thermal impact on product quality. 1st ed. London: Woodhead Publishing; 2008. p. 319-337.
8. Chen GQ, Lu FP, Du LX. Natamycin production by *Streptomyces gilvosporeus* based on statistical optimization. J Agric Food Chem. 2008; 56(13): 5057-61.
9. Institute of Standard and Industrial Researchs of Iran. Plain Doogh: characteristics and test methods, Iran National Standard, No. 2453, 2008b [In Persian].
10. Esfandiari Z, Badiey M, Mahmoodian P, Sarhangpour R, Yazdani E, Mirlohi M. Simultaneous determination of sodium benzoate, potassium sorbate and natamycin content in Iranian yoghurt drink (Doogh) and the associated risk of their intake through Doogh consumption. Iran J Public Health. 2013; 42(8): 915-20.
11. Dzigbordi B, Adubofuor J, Faustina Dufie WM. The effects of different concentrations of natamycin and the point of addition on some physicochemical and microbial properties of vanilla-flavored yoghurt under refrigerated condition. Int Food Res J. 2013; 20(6): 3287-92.
12. Sara AE, Ekbal MAI, Adham MA, Hamdi AM. The role of natamycin fortification to extend shelf life of plain yoghurt. Benha Vet Med J. 2014; 27(2): 140-9.
13. Tamime AY, editor. Fermented milks.1sted. New Jersey: John Wiley & Sons; 2008.
14. Ledenbach LH, Marshall RT. Microbiological spoilage of dairy products. In: Compendium of the microbiological spoilage of foods and beverages. New York: Springer; 2009. p. 41-67.
15. Pal M, Mulu S, Tekle M, Pintoo SV, Prajapati J. Bacterial contamination of dairy products. Beverage and Food World. 2016; 43(9): 40-3.
16. Institute of Standard and Industrial Researchs of Iran. Food Chain Microbiology- A Comprehensive Method for Counting microorganisms- Part 1: Colony Counting at 30° C using pour plate method- Iran National Standard, No. 5272-1, 2014 [In Persian].
17. Institute of Standard and Industrial Researchs of Iran. Microbiology of food and animal feed - A Comprehensive Method for Counting mold and Yeast - Part 1: Colony Counting Method in Products with water Activity More than 0.95, Iran National Standard, No. 10899-1, 2008a [In Persian].
18. Institute of Standard and Industrial Researchs of Iran. Microbiology of food and animal feed - Comprehensive method for counting coliform bacteria - colony counting method, Iran National Standard, No. 9263, 2007 [In Persian].
19. Institute of Standard and Industrial Researchs of Iran. Microbiology of food and animal feed - search and counting of Escherichia coli using the most probable number method, Iran National Standard, No. 2946, 2005a [In Persian].
20. Institute of Standard and Industrial Researchs of Iran. Microbiology of food and animal feed - Enumeration of Staphylococcus positive coagulase (*Staphylococcus aureus* and other species) - Test method, Part 1: Method of using Bird-Parker agar medium culture, Iran National Standard, No. 6806-1, 2005b [In Persian].
21. Institute of Standard and Industrial Researchs of Iran. Enumeration of mesophilic lactic acid bacteria by colony counting at 30 °C in food, Iran National Standard, No. 4721, 1998 [In Persian].
22. Adebayo CO, Aderiye BI, Akpor OB. Assessment of bacterial and fungal spoilage of some Nigerian fermented and unfermented foods. African Journal of Food Science. 2014; 8(3): 140-7.

23. Branen AL, Davidson PM, Thorngate J, Salminen S. editors. Food Additives. 2nd ed. London: CRC Press; 2001.

24. Gourama H, Bullerman LB. Effects of potassium sorbate and natamycin on growth and penicillic acid production by Aspergillus ochraceus. J Food Prot. 1988; 51(2): 139-44.

25. Jay JM, Loessner MJ, Golden DA. Modern food microbiology. 7thed. New York: Springer Science & Business Media; 2008.

26. Wouters JTM, Ayad EHE, Hugenholtz J, Smit G. Microbes from raw milk for fermented dairy products. Int Dairy J. 2002; 12(2-3): 91-109.

27. Yeganehzad S, Mazaheri-Tehrani M, Shahidi F. Studying microbial, physiochemical and sensory properties of directly concentrated probiotic yoghurt. Afr J Agric Res. 2007; 2(8): 366-9.

28. Akpan UG, Mohammed AD, Aminu I. Effect of preservative on the shelf life of yoghurt produced from soya beans milk. Leonardo Electronic Journal of Practices and Technologies. 2007; 11: 131-42.

29. Viljoen BC, Lourens-Hattingh A, Ikalafeng B, Peter G. Temperature abuse initiating yeast growth in yoghurt. Food Res Int. 2003; 36(2): 193-7.

30. Fleet GH. Yeasts in dairy products. J Appl Bact. 1990; 68(3): 199-211.

31. Sauer M, Porro D, Mattanovich D, Branduardi P. Microbial production of organic acids: expanding the markets. Trends Biotechnol. 2008; 26(2): 100-8.

32. Hervert CJ, Alles AS, Martin NH, Boor KJ, Wiedmann M. Evaluation of different methods to detect microbial hygiene indicators relevant in the dairy industry. J Dairy Sci. 2016; 99(9): 7033-42.

33. Rawat S. Food Spoilage: Microorganisms and their prevention. Asian Journal of Plant Science and Research. 2015; 5(4): 47-56.

34. Frank JF. In: Doyle MP, Beuchat LR, Montville TJ, editors. Food microbiology: fundamentals and frontiers. 2nd ed. Washington, DC: Am. Soc. Microbiol, 2001. p. 111–126.

35. Bachrouri M, Quinto EJ, Mora MT. Survival of Escherichia coli O157: H7 during storage of yogurt at different temperatures. J Food Sci. 2002; 67(5): 1899-903.

36. Rattanachaikunsopon P, Phumkhachorn P. Lactic acid bacteria: their antimicrobial compounds and their uses in food production. Ann Biol Res. 2010; 1(4): 218-28.